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(54) Title: DIAGNOSIS OF RETINOBLASTOMA (57) Abstract This invention relates to nucleic acid, or fragments thereof, encoding the retinoblastoma polypeptide, the retinoblastoma polypeptide itself, methods of detecting a defective retinoblastoma gene in human patients, and methods of treating these patients.		

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DIAGNOSIS OF RETINOBLASTOMA

Background of the Invention

This application is a continuation-in-part of Dryja et al. U.S.S.N. 146,525, filed January 21, 1988, which is a continuation-in-part of Dryja et al. U.S.S.N. 895,163, filed August 11, 1986, both hereby incorporated by reference.

This invention concerns the retinoblastoma gene and methods for detecting and treating patients afflicted with a defective retinoblastoma gene.

Retinoblastoma is a neoplastic condition of the retinal cells, observed almost exclusively in children between the ages of 0 and 4 years. It affects between 1 in 34,000 and 1 in 15,000 live births in the United States. (L.E. Zimmerman, 1985, Retinoblastoma and retinocytoma, In W.H. Spencer (ed.), Ophthalmic Pathology: an Atlas and Textbook, Vol. II, Philadelphia: W.B. Saunders Co., pp. 1292-1351.) If untreated, the malignant neoplastic retinal cells in the intraocular tumor travel to other parts of the body, forming foci of uncontrolled growth which are always fatal. The current treatment for a retinoblastoma is enucleation of the affected eye if the intraocular tumor is large; for small intraocular tumors, radiation therapy, laser therapy, or cryotherapy is preferred. There is no known successful treatment for metastatic retinoblastoma. As with most cancers, morbidity and mortality are reduced if diagnosis can be made early in the course of the disease.

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In 30-40% of cases of retinoblastoma, the affected individual carries a heritable predisposition to retinoblastoma and can transmit this predisposition to his or her offspring as a dominant trait (A.G. Knudson, 1971, Mutation and cancer: Statistical study of retinoblastoma, Proc. Natl. Acad. Sci., Vol. 68, pp. 820-23). Carriers of this retinoblastoma-predisposing trait are at a greatly elevated risk for development of several other forms of primary cancer, notably osteosarcoma and soft-tissue sarcoma.

The genetic locus associated with familial retinoblastoma has been assigned to the q14 band of human chromosome 13 (R.S. Sparkes et al., 1980, Science, Vol. 208, pp. 1042-44). Most retinoblastomas arise from cells which have lost both normal, dominant, homologous alleles at this retinoblastoma locus. However, individuals carrying one defective allele may be predisposed to the disease. Children who have had one eye affected by retinoblastoma or who are related to someone with retinoblastoma may be genetically predisposed and therefore at risk of developing the disease. These individuals routinely are tested for retinoblastoma every 2-3 months by an ocular examination procedure which requires placing the child under general anesthesia.

Summary of the Invention

In general, the invention concerns purified nucleic acid (less than 100kb in size), and fragments thereof of at

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least 15 bases, encoding the Rb gene. The invention also concerns cells transformed with this nucleic acid, isolated polypeptides encoded by this nucleic acid, and antibodies to this polypeptide, or to naturally occurring retinoblastoma polypeptide. Retinoblastoma polypeptide is the polypeptide encoded by the Rb gene. Further, the invention concerns a composition, suitable for treating a human having a defective Rb gene, containing retinoblastoma polypeptide, or a fragment thereof, in a pharmacologically acceptable carrier.

The invention also features methods of screening human patients to determine those not at risk of developing retinoblastoma and thus not requiring conventional examinations to be performed. This screening involves, for example, comparing nucleic acid of a patient with purified nucleic acid encoding a human Rb gene, or fragments thereof.

Thus in various aspects, the invention features methods of analyzing the predisposition of patients to retinoblastoma which involves detecting large and small deletions or point mutations in the retinoblastoma gene, or detecting the co-inheritance of such defects with specific restriction fragment length polymorphisms (RFLPs), or detecting the presence or absence of a normal or defective retinoblastoma gene by hybridizing a nucleic acid sample from the patient with a probe specific for the retinoblastoma gene, and determining the ability of the probe to hybridize to the nucleic acid. The

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lack of hybridization to the nucleic acid indicates the presence of a large deletion in the gene. A probe specific for the retinoblastoma gene may be hybridized to fragments separated by a defined physical property from a sample of a patient, the hybrids of the probe and the fragments detected, and the hybrids compared to hybrids detected from the hybridization of the probe and separated nucleic acid fragments from a normal retinoblastoma gene. The absence of hybrids or presence of hybrids of a smaller size compared to a normal patient is an indication of large deletions in the retinoblastoma gene of the patient. Preferably, the probe specific for the retinoblastoma gene is the cloned DNA in p4.7R; or a fragment thereof; and the defined physical property is molecular weight.

Small deletions or point mutations can be detected by determining the nucleotide sequence of a retinoblastoma allele from a patient, and comparing the nucleotide sequence with the nucleotide sequence of a retinoblastoma allele, or subregion thereof, from a person not afflicted with retinoblastoma; or by detecting mismatches between a nucleic acid sample from a patient and a probe specific for the retinoblastoma gene from a person not afflicted with retinoblastoma. The co-inheritance of specific genetic polymorphisms with the retinoblastoma gene may be an indication of the predisposition of a patient to retinoblastoma. According to this method, nucleic acid

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fragments are generated from a sample of the patient, the fragments are separated according to a defined physical property of the fragments (e.g., molecular weight), a detectable probe specific for the retinoblastoma gene is hybridized to the fragments, hybrids of the probe and the fragments are detected, and the hybrids are compared to hybrids detected from the hybridization of the same probe and separated nucleic acid fragments from a sample of a parent of the patient.

In another aspect, the invention features the use of an isolated normal human retinoblastoma gene to synthesize Rb polypeptide for use in the treatment of individuals determined to have a defective Rb allele.

In yet another aspect, the invention features a method of detecting the presence of the retinoblastoma polypeptide in a tumor sample from a human patient, by producing an antibody to the retinoblastoma polypeptide, contacting the antibody with the tumor sample, and detecting immune complexes as an indication of the presence in the tumor sample of the retinoblastoma polypeptide. The absence of the polypeptide indicates that the tumor is caused by a defect in a retinoblastoma allele. This procedure would preferably involve contacting a tumor sample from a human patient with an antibody (e.g., monoclonal antibody) which specifically reacts with the retinoblastoma polypeptide, or a fragment thereof, and determining whether the antibody binds to cells of the tissue

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specimens. The absence of immune complexes is an indication that the tumor was the result of a defective retinoblastoma allele.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The description of the preferred embodiments of the invention will follow the brief description of the drawings given below.

Drawings

Fig. 1 is a diagrammatic representation of a restriction map of the insert in the clone p4.7R.

Fig. 2 is a diagrammatic representation of a restriction map of the genomic locus of the retinoblastoma gene.

Fig. 3 is a diagrammatic representation of the vectors p2AR3.8 and p2AR0.9 of the invention.

Fig. 4 is a scale map of the normal retinoblastoma gene.

Fig. 5 (5-1 through 5-3) is a nucleic acid sequence of a cDNA of the normal retinoblastoma gene, with flanking regions.

Fig. 6 (6-1 through 6-9) is a nucleic acid sequence of exons of the normal retinoblastoma gene, with flanking regions.

Fig. 7 is a restriction map of the retinoblastoma gene, showing the locations of DSPs.

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Fig. 8 is a gel and a diagram showing the inheritance of the polymorphism RB1.3 in a retinoblastoma-prone family.

Fig. 9 is a diagram showing the segregation of the DSP RB1.3 in three families with hereditary retinoblastoma.

Retinoblastoma Polypeptide

The Rb polypeptide is the specific amino acid chain encoded by the nucleic acid sequence of the normal retinoblastoma gene. The Rb polypeptide of this invention includes: (1) naturally occurring retinoblastoma protein; (2) synthetically produced retinoblastoma polypeptide; and (3) retinoblastoma polypeptide produced from purified nucleic acid (e.g., cDNA or genomic DNA) via an in vitro expression system. Also included are biologically active fragments of Rb polypeptide which either have a biological activity of naturally occurring Rb polypeptide, or include an epitope of this polypeptide and thus are suitable for production of Rb-specific antibodies.

Retinoblastoma Gene

The Rb gene is that distinct nucleic acid sequence in the human genome, the absence or mutation of which predisposes one to retinoblastoma. The purified nucleic acid sequence encoding the retinoblastoma gene can be carried on vectors which can be propagated in cells. For the purposes of this invention, purified nucleic acid encoding the Rb gene is defined as nucleic acid isolated from its natural environment

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(e.g., cDNA or a fragment of genomic DNA) which hybridizes specifically to the retinoblastoma gene under hybridizing conditions. An example of purified nucleic acid which encodes the retinoblastoma gene, and is carried on a vector, is the cDNA clone p4.7R. This clone was obtained in the following manner.

cDNA

The human DNA probe pH3-8, isolated from a human chromosome 13 lambda phage library (M. Lalande et al., 1984, Cancer Genet. Cytogenet., Vol. 13, pp. 283-95), was used in a chromosome walking technique to isolate and map 30 kilobases (kb) of genomic DNA surrounding the H3-8 sequence. One fragment generated by this technique, named p7H30.7R, was found to recognize a DNA sequence in the mouse genome as well as one within human chromosome 13 (T.P. Dryja et al., 1986, Proc. Natl. Acad. Sci. USA, Vol. 83, pp. 7391-94). The homology of p7H30.7R to both human and mouse DNA suggested that p7H30.7R contains coding sequences of a structural gene.

To test this possibility, p7H30.7R was radiolabeled and used to probe a Northern blot of RNA isolated from three retinoblastoma tumors and an adenovirus 12-transformed human embryonic retinal cell line (Vaessen et al., 1986, EMBO Journal, Vol. 5, pp. 335-). The p7H30.7R probe hybridized to an RNA transcript of approximately 4.7 kb from the retinal cell line, but did not hybridize to any RNA transcripts from the

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three tumor samples.

Subsequently, RNA isolated from the adenovirus-transformed retinal cell line was used to construct a cDNA library. This library was screened with the labeled p7H30.7R probe. Several cDNA clones were isolated which had similar restriction maps. The longest of these, p4.7R, contained 4.7 kb of DNA. The restriction map of the insert in the clone p4.7R is shown in Fig. 1.

The p4.7R clone was used to screen RNA transcripts isolated from four retinoblastomas, an osteosarcoma, and the adenovirus-transformed retinal cells. In a Northern blot analysis of isolated RNA's, the p4.7R probe cross-reacted with a 4.7 kb transcript in the transformed retinal cells which was not present in the four retinoblastoma and one osteosarcoma cell samples.

Genomic DNA

Clones containing genomic DNA including the retinoblastoma gene were isolated in the manner described below. Recombinant bacteriophage libraries containing human genomic DNA fragments inserted in the lambda phage cloning vector EMBL-3 were constructed according to published methods (Seed et al., 1982, Gene, Vol. 19, pp. 201-209). Those recombinant bacteriophage which contain fragments of the retinoblastoma gene were initially detected by hybridization of the bacteriophage plaques with p4.7R.

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Thirty-six distinct recombinant bacteriophage that contain overlapping human genomic DNA fragments were isolated. Selected bacteriophage were plaque-purified and amplified, and the restriction map of each phage insert was determined by the method of Rackwitz et al., Gene, Vol. 30, pp. 195-200.

With these bacteriophage a restriction map of a region that spans approximately 200 kb was constructed, shown in Figs. 2 and 4. All of the known sequences present in the mRNA from the retinoblastoma gene are present in this cloned region. In aggregate, the human DNA sequences in this set of bacteriophage represent the chromosomal segment of the Rb gene. In Fig. 2, the vertical marks above the map represent the location of HindIII sites, and the vertical marks below the map represent the location of EcoRI sites. The boxed areas represent HindIII fragments which contain sequences found in the cDNA (exons). Each double-headed arrow beneath the map represents a distinct recombinant bacteriophage clone. Fig. 4 shows all the recognition sites of the six restriction enzymes Hind III, EcoR I, Xba I, Sac I, Sac II, and BamH I (New England Biolabs, Inc.). Restriction endonuclease fragments that contained exons were identified by their hybridization with cDNA clones or synthesized oligonucleotide sequences based on cDNA sequence. These restriction fragments were subcloned in the plasmid vector Bluescribe (Stratagene, San Diego, CA). A total of 24 distinct plasmids were subcloned in this manner.

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The number and size of each exon was determined by iterations of the following procedure. First, an oligonucleotide was synthesized that corresponded to the first 20 nucleotides of the cDNA sequence. Using this oligonucleotide as a primer, the plasmid with a genomic insert containing the most 5' exon was sequenced. The resultant sequence was aligned with the cDNA sequence to determine the length of the first exon; the point at which the plasmid and cDNA sequences diverged marked the beginning of the first intron. This exon and the flanking regions were further sequenced using synthetic oligonucleotide primers to generate a continuous nucleotide sequence composed of 5' promoter sequence, exon 1, and the beginning of intron 1. The second and subsequent exons were defined by synthesizing sequencing primers corresponding to the next 20 nucleotides of cDNA sequence that had not been previously assigned to an exon. All exons and the immediately adjacent flanking intron sequences were sequenced in both sense and antisense directions.

The dideoxynucleotide chain termination method of sequencing was carried out using the enzyme Sequenase (United States Biochemical Corporation, Cleveland, Ohio) according to protocols supplied by the manufacturer. The intron region downstream of exon 20 could not be sequenced by this method, due to an unusually problematic repeated sequence in this region that caused a series of 45 stops (bands appeared in all

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four lanes of the sequencing gel). To resolve this region, sequencing reactions were carried out with Taq polymerase (Perkin-Elmer/Cetus). This enzyme allowed for the polymerization to be performed at 68°C and resolved the bases in this region.

All sequence data were analyzed and screened for overlapping regions using the sequence analysis program Microgenie Sequence Software (Beckman, Palo Alto, CA).

The position of each exon within the restriction map of the gene was determined by hybridization of cDNA fragments or synthetic oligomer sequences to recombinant bacteriophage DNA that had been digested with various restriction endonucleases. The precise location of most exons was subsequently deduced when recognition sequences of endonucleases were identified within the intron-exon sequence and correlated with the map. The position of each of the remaining exons was arbitrarily placed in the center of the smallest restriction fragment to which it hybridized.

The organization of the 27 exons along the genomic map of the retinoblastoma gene is illustrated in figure 4. This figure details the recognition sites for 6 restriction endonucleases and the position of the exons relative to these sites. Exons 1, 2, 3, 6, 9, 10, 13, 21, 22, 23, 24, 25, 26, and 27 have been precisely localized on this map. The other exons were mapped within small restriction fragments and are

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illustrated in the middle of these fragments. Exons 11, 17, and the cluster of exons 14-16 were mapped by this technique with uncertainties of not more than 2.0 kb. The remaining exons (exons 4, 5, 7, 8, 12, 18, 19, and 20) were all mapped to within 0.8 kb of their true locations. For reference, this map also shows the position of several naturally occurring restriction fragment length polymorphisms.

Fig. 6 (6-1 through 6-9) shows the sequence flanking and including each exon. The exons range in size from 31 nucleotides (exon 24), to 1973 nucleotides (exon 27). The shortest intron sequence was found to be 80 nucleotides long and is located between exons 15 and 16, whereas the largest spans approximately 70.5 kb between exon 17 and 18. All of the intron donor and acceptor splicing sites comply with the GT-AG splice junction rule. Our methods of sequencing proved more accurate than previous reports in defining the exact number of exons comprising the retinoblastoma gene.

The first exon and the region immediately 5' to this exon are very G-C rich, which is a characteristic of promoter regions. This region contains 9 possible Hpa II restriction sites, is composed of 66% C+G nucleotides and does not exhibit CpG suppression. These criteria are indicative of a HTF island. This promoter region contained some nucleotides that could not be resolved in either the sense or antisense direction using either Sequenase or Taq polymerase.

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Presumably, this was due to secondary structure that forms in this promoter region.

Analysis of the sequence approximately 30 nucleotides upstream of the transcription initiation site defined by Lee et al. (1987b) does not reveal a TATA box that is found in other promoter regions. This suggests that either the previously published initiation site is not in fact correctly defined, or that the retinoblastoma gene lacks the prototypical TATA and CAAT boxes of promoter regions. Further analysis of the sequence 5' to exon 1 reveals a possible TATA box at base pair #-274, labeled base pair #122 in figure 6-1. Homology for the seven base region is only 57%, yet the first four bases T-A-T-A, which are the bases most frequently conserved, are 100% homologous. A possible capping site, CAC, is located 14 bases away and again 49 bases away. CAAT boxes were not identified although the region is generally G-C rich.

The intron sequence that flanks the 3' side of exon 20 consisted of 21 consecutive repeats of the sequence TTT(T)C that together span 87 nucleotides. The number of repeat units can vary between different individuals and the alleles determined by the number of repeats behave like a heritable DNA polymorphism.

A computer search of the sequence data identified several intron regions homologous to Alu repetitive sequences. Alu repeats were located in the following regions: (1)

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downstream of exon 2, between bp 492 and bp 704 according to the numbering scheme in Figure 6; 2) upstream of exon 9, between bp 19 and bp 117; 3) downstream of exon 11, between bp 504 and bp 680; 4) the intron sequence flanking both sides of exon 14 between bp 132 and bp 270 and between bp 420 and bp 741; and, 5) upstream of exon 17, between bp 36 to bp 210. The Alu sequence located downstream of exon 2 contains two internal sequences that are highly conserved in Alu repetitive sequences. The first is a sequence (GAGGCNGAGC) corresponding to the T-antigen binding sequence of the SV40 replication origin. The second is a symmetrical sequence (CCAGCCTGG) of no known function. This short symmetrical sequence is also present in both of the Alu sequences on either side of exon 14. Exons 14 and 15 are separated by a short intron that is almost entirely composed of Alu sequence, suggesting that exons 14 and 15 were possibly at one time a single exon and were divided by the insertion of an Alu element during evolution. This Alu sequence may have been directed to this position by the other Alu sequence located on the 5' end of exon 14 because retroposons have a tendency to integrate adjacent to one another.

The 3' end of the retinoblastoma gene contains the usual polyadenylation signal sequence, AATAAA. One sequence (TGTGTTCT) located 32 bases downstream of this hexamer is equivalent to the conserved downstream consensus sequence

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(YGTGTYT) described by McLauchlan et al. (1985). This sequence and surrounding bases compose the "G/T cluster" generally found in a region 30 nucleotides downstream of the polyadenylation signal sequence (Birnstiel et al., 1985).

The p4.7R probe also was used to screen genomic DNA isolated from the tumors of 50 unrelated individuals (40 retinoblastomas, 8 osteosarcomas, and 2 undifferentiated tumors of unknown cellular origin arising in patients with hereditary retinoblastoma), as described in more detail below. These DNA samples were digested with HindIII and analyzed by Southern blot hybridization using radiolabeled p4.7R as the probe. This analysis revealed three types of deviant patterns of the genomic DNA restriction fragments: totally absent fragments, representing apparent homozygous deletions; under-represented fragments, representing apparent heterozygous deletions; and fragments of altered size, reflecting either partial deletion or an alteration of a restriction site. At least 30% of the tumor DNA's exhibited one of these abnormalities. In comparison, Southern blot analysis of leucocyte DNA from 18 normal individuals showed a uniform pattern of restriction fragments.

Use

The cDNA and genomic sequences, e.g., those in p4.7R, can be used, according to the invention, to screen individuals for the presence of a mutated allele of the Rb gene. This

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screening procedure will allow individuals having a risk of developing retinoblastoma--because of family history or a previous incidence of retinoblastoma in one eye--to determine the need for routine testing by the current ocular examination procedure. Only if the screening procedure determines that the individual possesses a mutant Rb allele will the examination procedure need to be conducted on a regular basis. Those with two normal Rb alleles can discontinue examination, as the risk of developing retinoblastoma in an individual with two normal copies of the Rb gene is approximately 1 in 20,000, or 0.005%, compared to a risk of 80%-90% if an individual has an Rb allele containing a mutation sufficient to inactivate the allele. Thus, a substantial percentage of individuals who are currently examined regularly are not actually at a greater risk than the general population: neither a family history of nor a previous incidence of retinoblastoma is conclusive evidence that an individual has the genetic predisposition to the disease. Therefore, such individuals, actually carrying two normal copies of the Rb gene, have been repeatedly undergoing the expensive and traumatic ocular examination procedure needlessly.

The screening procedure according to the invention includes: (1) testing a nucleic acid sample of a patient for large deletions in the Rb gene locus; (2) testing a nucleic acid sample of a patient for small deletions or point mutations in the Rb gene locus; and (3) testing a nucleic acid sample of

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a patient for RFLPs linked to the Rb gene locus.

Detection of Large Deletions in the Rb Gene

The availability of DNA probes from the Rb gene provides a means of directly detecting genetic lesions that create retinoblastoma-predisposing alleles. Suitable probes include the entire normal retinoblastoma gene sequence, or fragments thereof consisting of 15 or more bases encoding a specific portion of the retinoblastoma gene. When performed by Southern blot and dot blot procedures, this analysis is generally limited to the study of those lesions that create gross structural changes in the Rb gene, such as deletion of many hundreds of base pairs.

The DNA for a Southern Blot or dot blot analysis is isolated from peripheral leucocytes or, if the patient has had a tumor in one eye, from the tumor. To examine leucocyte DNA, a 10 ml blood sample is obtained from the individual, and the genomic DNA is isolated from the leucocytes in the sample, according to standard techniques. This DNA is digested with a restriction endonuclease (e.g., HindIII), and the resulting fragments are separated on an agarose electrophoresis gel according to a physical property such as molecular shape or molecular weight. For the purposes of this invention, molecular shape is defined as the structural configuration of the molecule (e.g., linear, circular, double-stranded or single-stranded). The DNA in the gel is transferred to a

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nitrocellulose filter by blotting. The filter is then probed with, e.g., radiolabeled p2AR3.8 and, separately, p2AR0.9, containing subfragments from p4.7R obtained by EcoRI digestion. (The diagrams of the vectors p2AR3.8 and p2AR0.9 are shown in Figure 3.) In order to more precisely define the location of any abnormalities detected, two or more subfragment probes are used separately rather than the entire p4.7R insert probe. The autoradiograms of the probed filter generate the data necessary to construct a restriction map of the Rb locus in the somatic or tumor DNA of the tested individual.

This restriction map is compared with a control restriction map, determined by using the same restriction enzymes for digestion and the same probe. A suitable control is DNA obtained from an adenovirus-transformed retinal cell line or leucocyte DNA from a set of normal individuals. If the tested individual has an Rb allele containing a significantly large deletion, a restriction map of his DNA, compared with the control, will contain an additional band or bands, and/or a band or bands that have lost 50% of their intensity, caused by a change in the size, or total elimination, of one or more restriction fragments by the deletion in one allele at the Rb locus.

This screening procedure by Southern analysis will detect the existence of Rb alleles which have large deletions and are thereby non-functional. If this analysis indicates

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that the tested DNA from an individual has a restriction map which is different from the control map, there is a high probability that the individual contains a non-functional, mutant Rb allele. The individual must be monitored closely thereafter for the development of retinoblastoma.

If the test restriction map appears identical to the control, a different screening procedure can be performed to determine if the individual possesses an Rb allele having a small deletion or point mutation. Small deletions and point mutations may be sufficient to inactivate the allele, but not prevent hybridization with a probe. An example of this screening procedure is outlined below.

Detection of Other Mutations in the Rb Gene

To examine a DNA sample of an individual for small deletions or point mutations in the Rb locus, both homologs of the Rb gene from said individual are cloned. The cloned alleles then can be tested for the presence of nucleic acid sequence differences from the normal allele, e.g., as represented by p4.7R, by one of the following two methods: (1) the nucleotide sequence of both the cloned alleles and p4.7R are determined and then compared, or (2) the RNA transcripts from p4.7R are hybridized to single stranded whole genomic DNA from an individual to be tested, and the resulting heteroduplex is treated with Ribonuclease A (RNase A) and run on a denaturing gel to detect the location of any mismatches. In

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more detail, these methods can be carried out according to the following procedure.

The alleles of the Rb gene in an individual to be tested are cloned using conventional techniques. A common method, for example, employs the bacteriophage vector EMBL3 (Frischauf et al., 1983, J. Mol. Biol., Vol. 170, pp. 827-). A 10 ml blood sample is obtained from the individual. The genomic DNA isolated from the cells in this sample is partially digested with MboI to an average fragment size of approximately 20 kb. Fragments in the range from 18-21 kb are isolated. The resulting MboI-ended fragments are ligated into the EMBL3 vector DNA which has been completely digested with BamHI, treated with alkaline phosphatase, and heated to 68°C for 10 minutes to disrupt the cohesive ends. This ligation mix is used in an in vitro lambda packaging reaction, and the packaged phage are amplified by growing a plate stock. (This cloning technique is described generally in Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Publications, pp. 256-293.)

Approximately 5×10^5 plaque forming units (pfu) from this plate stock are then screened with radiolabeled p4.7R by hybridization and autoradiography. Plaques which show hybridization to the p4.7R probe are plaque-purified and rescreened according to the above procedure. Positive plaques from the rescreening are isolated and used to prepare DNA

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putatively containing Rb alleles from the individual.

The MboI genomic inserts in these isolated EMBL3 vector DNA samples are tested for the location of the sequences homologous to p4.7R by Southern analysis. DNA samples containing the entire Rb gene region are selected, and the appropriate restriction fragments containing the Rb gene from these samples are subcloned into a suitable vector, such as pUC9. These subclones thus contain copies of one or both Rb alleles from the DNA of the individual to be tested. To determine if both alleles are represented, the initial phage isolates are tested for the existence of restriction polymorphisms. These subcloned alleles are then examined for differences from p4.7R by one of the following techniques.

First, the nucleotide sequence of the normal Rb gene in p4.7R is determined. Restriction fragments of approximately 500 base pairs (bp) from p4.7R are subcloned into an M13mp8 phage vector and sequenced by the dideoxy technique (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA, Vol. 74, pp. 5463-). A composite sequence of the Rb gene then can be assembled from these individual subclone sequences. The complete sequence of the normal retinoblastoma gene and flanking sequences is shown in Fig. 5 and Fig. 6.

The isolated Rb gene alleles are sequenced according to the following procedure. Restriction fragments (~ 2kb) of the allele are subcloned into the M13mp8 vector, and short

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stretches (~500 bp) are sequenced individually using small restriction fragments isolated from p4.7R as the primers in the dideoxy sequencing reactions. The composite nucleotide sequence of the isolated allele then can be constructed from these individually-primed sequences. This sequence is compared directly with the sequence of the normal Rb gene, determined from p4.7R, to reveal any deletions or point mutations in the isolated allele.

An alternative method of comparing the allelic DNA with the normal Rb gene employs RNase A to assist in the detection of differences between the p4.7R sequence and the allele sequence. This comparison is performed in steps using small (~500 bp) restriction fragments of p4.7R as the probe. First, p4.7R is digested with a restriction enzyme(s) that cuts the Rb gene sequence into fragments of approximately 500bp. These fragments are separated on an electrophoresis gel, purified from the gel and cloned individually, in both orientations, into an SP6 vector (e.g., pSP64 or pSP65; Melton et al., 1984, Nucleic Acids Res., Vol. 12, pp. 7035-). The SP6-based plasmids containing inserts of p4.7R fragments are transcribed in vitro using the SP6 transcription system, well known in the art, in the presence of [α - 32 P]GTP, generating radiolabeled RNA transcripts of both strands of the cDNA of the Rb gene.

Individually, these RNA transcripts are used to form

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heteroduplexes with the allelic DNA, as described by Myers et al., 1985, Science, Vol. 230, pp. 1242-46, the teachings of which are incorporated herein by reference. Mismatches that occur in the RNA:DNA heteroduplex, owing to sequence differences between the p4.7R fragment and the Rb allele subclone from the individual, result in cleavage in the RNA strand when treated with RNase A. Such mismatches can be the result of point mutations or small deletions in the individual's Rb allele. Cleavage of the RNA strand yields two or more small RNA fragments, which run faster on the denaturing gel than the RNA probe itself.

In the RNase A technique, radiolabeled Rb gene RNA is hybridized to single strands of an individual's Rb alleles which have been cloned into a vector. The RNase A technique is advantageous, however, because it also can be used without having to clone the Rb alleles. Preferably, genomic DNA is isolated from blood cells of the individual to be tested, and this genomic DNA is hybridized directly with the radiolabeled Rb RNA probes to determine sequence differences from the normal Rb gene. Specifically, 5 µg of isolated, total genomic DNA is resuspended with the labeled RNA probe in 30 µl of hybridization buffer (80% formamide, 40mM Pipes pH6.4, 0.4M NaCl, and 1mM EDTA), and this hybridization mix is treated at 90°C for 10 minutes to denature the DNA. The mixture then is cooled slowly to 45°C and incubated at this temperature for 10

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hours to allow hybridization of the RNA probe to the single-stranded DNA copies of the Rb allele. After hybridization, RNase A treatment and electrophoresis are performed as described by Myers et al., supra. Mismatches between the RNA probe and the genomic copies of the individual's Rb alleles are then readily detected.

Detection of RFLPs Linked to the Rb Gene

The inheritance of a retinoblastoma- predisposing defect can be traced by following its co-inheritance with DNA polymorphisms in a pedigree analysis.

The gene map shown in Figure 2 was used to develop nucleic acid probes useful for retinoblastoma diagnosis. To do so, the bacteriophage DNA corresponding to the human inserts were subcloned in the plasmid vector "Bluescribe" (Stratagene). Fifteen single-copy DNA fragments from the gene, ranging in size from 500 bp to 2000 bp, were subcloned. These sequences are scattered over the 200 kb of the mapped region. Subcloned DNA fragments were separated from vector sequences by digestion of plasmid DNA with one or more restriction endonucleases, electrophoresis through a 0.6% low-melting-point agarose gel, and purification by chromatography using an Elutip-d column (Schleicher and Schuell). Purified DNA fragments were radiolabeled with ^{32}P -dCTP (New England Nuclear) by the random primer technique using the Klenow fragment of DNA polymerase I.

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Restriction fragment length polymorphisms (RFLP's) were discovered by digesting genomic DNA isolated from six normal individuals with 33 different restriction enzymes. The DNA fragments resulting from the 198 separate digests were separated on a 0.8% agarose electrophoresis gel according to molecular shape or molecular weight. The DNA was transferred to nitrocellulose filters and hybridized with single copy DNA probes purified from the retinoblastoma gene according to published methods (T.P. Dryja et al., 1986, Proc. Natl. Acad. Sci. USA, Vol. 83, pp. 7391-94).

Of the 15 single-copy probe fragments, only five reveal RFLP's. Four of the polymorphisms appear to be the result of minor alterations (perhaps single base changes) in the recognition sequence of a restriction endonuclease (KpnI, XbaI, MboII, or Tth111I). The fifth polymorphism reflects variability in the number of tandem repeats of a 50 base pair sequence. The location of the DNA polymorphisms are shown in the map in Figure 2 (vertical arrows above the map). The location of the polymorphic MboII site(s) has not been determined precisely but is located at approximately 175 kb on this map. The frequencies of alleles which correspond to particular DNA polymorphisms are indicated in Table 1.

In order to demonstrate the utility of these probes to detect the presence of retinoblastoma-predisposing alleles in humans, twenty pedigrees with hereditary retinoblastoma were

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analyzed. DNA was extracted from leucocyte nuclei of venous blood from available family members according to the method of Kunkel et al., 1977, Proc. Natl. Acad. Sci. USA, Vol. 74, pp. 1245-49, hereby incorporated by reference. For analysis of a kindred with a given RFLP, DNA from the available family members was digested with the appropriate restriction endonuclease. The resulting fragments were separated by agarose-gel electrophoresis, transferred to nitrocellulose filters, and hybridized to labeled probe.

In these families, the inheritance of alleles determined by the DNA polymorphisms within the retinoblastoma gene were traced and compared to the inheritance of the retinoblastoma-predisposing trait. For example, consider the polymorphism detected by probe p68RS2.0 (see Table 1). When genomic DNA is digested with the restriction enzyme RsaI, this probe hybridizes to allelic DNA fragments of different lengths. The size of these fragments ranges from 1.5 kb to 2.0 kb with intervals of approximately 50 bp. The DNA sequence of the 2.0 kb genomic fragment cloned in p68RS2.0 has a 50 to 53 bp segment which is repeated approximately 30 times (Table 2). This 53bp segment can be used as a probe in these analyses. A portion of the repeated sequence has homology to core sequences of VNTR's (Variable Number of Tandem Repeat) reported elsewhere (Y. Nakamura et al., 1987, Science, Vol. 235, pp. 1616-22). (The 11 bp sequence shown in Table 2 above the repeat unit

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TABLE 1					
DNA POLYMORPHISMS					
IDENTIFIED WITHIN THE RETINOBLASTOMA GENE					
DNA PROBE	RESTRICTION ENDONUCLEASE	MAP LOCATION ¹	ALLELES SIZE (Kb) ³	FREQUENCY ⁴	
p68RS2.0	<u>Rsa</u> I	142-143 kb	2.00	0.13	
			1.95	0.02	
			1.90	0.07	
			1.85	0.07	
			1.80	0.35	
			1.75	0.20	
			1.65	0.09	
			1.50	0.09	
p88PR0.6 ⁵	<u>Xba</u> I	120 kb	7.0	0.55	
			5.5	0.45	
p35R0.6	<u>Tth</u> 111I	195 kb	4.95	0.20	
			4.35	0.80	
p2P0.3	<u>Mbo</u> II	175 kb ²	1.0	>.90	
			0.8	<.05	
			0.6	<.05	
			0.3	<.05	
p95HS0.5	<u>Kpn</u> I	25 kb	12.0	0.95	
			8.0	0.05	

¹The map location of each polymorphic site refers to the position on the restriction map of the gene shown in Figure 1.

²The location of the MboII site is approximate, since the precise position of this site within the map is not yet known.

³The allele sizes were calculated from several independent measurements using HindIII fragments of lambda phage DNA as a standard.

⁴Allele frequencies are based on a population of 40-60 unrelated individuals.

⁵p88PRO.6, p35RO.6, p2PO.3 and p95HS0.5 are probes isolated from other regions of the retinoblastoma gene, shown in Fig. 2.

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represents the core sequence reported for some VNTR's observed by Nakamura et al.) Because such tandemly repeated sequences tend to be genetically unstable, the number of repeats is highly variable. Eight distinct alleles at this site have been detected, and more may exist. Because of this number of common alleles, seventy-five percent of unrelated individuals are heterozygous for this polymorphism. The high frequency of heterozygosity makes this polymorphism extremely useful. (In Table 2, the brackets "(" and ")" denote regions of variability within the repeat unit; and the bases underlined above and below the bracketed regions denote possible alternate bases for those regions of variability.)

TABLE 2
SEQUENCE OF THE REPEAT UNIT WITHIN p68RS2.0

GGGNNGTGGGG

CGTGTTGAA(TG)ACAC(A)TCCCAATGCTGGAGGTGAGGTTTGGT(AG)ATGACT

G

Fourteen retinoblastoma families carried constellations of alleles at this DNA polymorphism. This variation allows an examination of the frequency of co-inheritance of this site with the retinoblastoma-predisposing trait. For example, genomic DNA of individuals in these families was digested with Rsa I and the co-inheritance of any one Rsa I fragment with defective Rb alleles determined.

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Sequence Analysis

RFLP analysis can reveal only those sequence variations that give rise to a restriction fragment that is detectably different on a Southern blot from restriction fragments characterizing the normal nucleic acid. Most such detectable polymorphisms result from DNA sequence variation within a restriction endonuclease recognition site. These sites are rare, and finding them requires a laborious and expensive screening process in which genomic DNAs from several unrelated individuals are digested with as many as 50 different restriction enzymes. The fraction of all genomic DNA sequence polymorphisms (DSPs) at a specific locus that can be detected as RFLPs is small and depends on the number of enzymes used for screening; generally 90% or more of the DNA sequence polymorphism in the human genome is not within reach of RFLP-based analysis. Tens or even hundreds of potentially useful DSPs may exist within or near most disease-causing genes, but often only a few and sometimes none of these DSPs are detectable as RFLPs. Fig. 4 shows a scale map of the 200 kilobase genomic region that includes the 27 exons of the human retinoblastoma gene. The 27 exons make up a 4.7 kilobase transcript.

Fig. 7 shows the locations of the DNA sequence polymorphisms (DSPs) identified in this gene. Polymorphisms identified by the name of a restriction enzyme are RFLPs;

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polymorphisms RB1.2, RB1.3, RB1.20 and RB1.26 are not detectable as RFLPs, and were found by PCR-amplification and direct sequencing, as described below. The 200 kilobase genomic region was isolated in a series of overlapping inserts from 35 distinct recombinant bacteriophage lambda clones. Exon-containing segments were subcloned into bluescribe plasmid cloning vectors (Stratagene, Inc.). Initial sequencing of cloned plasmid inserts was carried out using conventional methods for plasmid sequencing. Based on this genomic sequence, pairs of 20-base oligonucleotide primers were synthesized so that numerous regions 320 - 1200 bp in size could be amplified from genomic DNA by the polymerase chain reaction (PCR) method of Mullis et al.

For each amplification reaction, from 200 ng to 1.0 ug of genomic DNA was prepared in a reaction buffer containing 20 mM Tris (pH 8.4 or pH 8.6), 30 ug/ml bovine serum albumin, 300 mM to 7.5 mM, 10-50 pM of each oligonucleotide primer, and 1 unit of Taq polymerase (Perkin-Elmer Cetus). Optimal MgCl₂ concentrations and pH of the PCR reactions varied depending on the primer pair. PCR-amplification (30-35 rounds) was carried out following a cycle of 10 seconds at 94°C (denaturation), 10 seconds at 42-55°C (annealing), and 30 seconds at 70°C (Polymerization) using a programmable thermal cycler (Ericomp Corp., San Diego). All times are based on sample temperature rather than heat-block temperature, and do not include 'ramping

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time' for the heat block. Optimal annealing temperatures varied for each primer pair. This protocol allowed PCR-amplification of regions as large as 2500 bp. Genomic DNA from 9-20 individuals was amplified for each region and screened for DSP.

In order to detect and utilize a greater fraction of the existing DNA sequence polymorphism in the retinoblastoma gene, we applied techniques of polymerase chain reaction (PCR), generally as described by K.B. Mullis et al., 1987, Methods Enzymol., Vol. 155, pp. 335-51, and direct sequencing, generally as described by C. Wong et al., 1987, Nature, Vol. 330, 384-86, to analyse normal allelic variation at this locus. Oligonucleotide primers were synthesized to amplify regions from the gene that varied in size from 320 - 1200 bp. Amplification and sequencing were carried out on DNA from at least 9 unrelated individuals for all regions screened, though for many regions 15 or more individuals were analyzed. In most cases, primer pairs were derived from intronic sequences that flanked one of the 27 exons of the gene, such that the PCR-amplified region contained both intron and exon sequences.

The results of this screening process are shown in Tables 3 and 4. Amplified DNA sequences were compared to one another and checked against sequence data from previously cloned plasmid inserts derived from the same region. Bases obscured by technical artifacts or other ambiguities were not

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tabulated. Of 3712 bp of genomic DNA sequence screened at this locus, four sequence variations were identified (Table 3; map locations are shown in Fig. 7). All four variations were found in introns; of these, one is likely a rare variant (found in only 1 or 15 individuals sequenced), and three represent bona fide DNA sequence polymorphisms. A representative example (RB1.3) is illustrated in Fig. 7. This polymorphism occurs near exon 3 of the retinoblastoma gene. Neither form of the polymorphic sequence forms the recognition site of a known restriction enzyme, and hence this DSP is not detectable as an RFLP. Among a total of 82 genetically distinct (from unrelated individuals) alleles examined, no other base was observed at this site.

Fig. 8 illustrates the inheritance of the polymorphism RB1.3 in a retinoblastoma-prone family. Oligonucleotide primers (see Table 4) were used to PCR-amplify a 530 bp region of the human retinoblastoma gene that includes exon 3. The amplified fragment was sequenced by the methods described below. The sequence surrounding the polymorphism is written at the left side of the figure, read 5' to 3' from bottom to top, and the polymorphic bases are identified by adjacent tic marks.

The details of the analysis were as follows. Prior to sequencing, all PCR-amplified DNA samples were treated with proteinase-K and extracted with phenol/chloroform. High molecular weight DNA was separated from unused dNTPs and

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oligonucleotide primers by column purification through sepharose CL-6B (Pharmacia). 250-400 ng of double-stranded PCR-amplified template was combined with 1-2 pM of (32 P) end-labeled sequencing primer, and heat-denatured for 3 minutes at 96°C. This primer-template mixture was added to a buffer containing: MgCL₂ (2.5 mM), Tris-HCL pH 7.5 (5 mM), 6 units Sequenase (U.S. Biochemical) and dithiothreitol (3 mM), and divided into 4 reaction mixtures each containing all four deoxynucleotides (32 uM each) and one dideoxynucleotide (5 uM). This mixture was immediately incubated for 5 minutes at 37-42°C, and polymerization was stopped with a 0.37% EDTA stop buffer. Prior to loading on sequencing gels, the samples were heat denatured at 96°C for 2 minutes. Conventional 0.4 mm thick, 6% polyacrylamide sequencing gels were used, and autoradiography was typically for 12-24 hours without an intensifying screen.

Fig. 9 illustrates segregation of the DSP RB1.3 in three families with hereditary retinoblastoma. Alleles are shown beneath the symbol for each person. Affected individuals are indicated by filled symbols. In family RB-32, the (-) allele is the result of an intragenic deletion. By subsequent Southern blotting studies, the deletion was found to extend from exon 2 to exon 17. Based on these results, it can be predicted that the unaffected members of family RB-32 who carry the (G,-) genotype are also carriers of the mutation.

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The DSPs we have detected are valuable genetic markers for our studies of hereditary retinoblastoma. In its hereditary form, a predisposition to the disease is passed from affected individuals to their offspring as a dominant trait with 90% penetrance. It can be seen from Fig. 8 that the affected father, who has passed the disease to two children, is heterozygous for RB1.3. Both affected children received the G allele, while the unaffected child inherited the allele marked by an A at this polymorphic site. In this family, then, inheritance of the G allele from the affected parent is in phase with and diagnostic for the disease-predisposing phenotype. Fig. 9 shows our analysis of three other retinoblastoma-prone families using RB1.3. Inheritance of the polymorphic markers we describe here has followed the expected Mendelian pattern in every family examined so far. No cross-overs were observed between the polymorphic sites and the retinoblastoma-predisposing trait in any of the pedigrees. This follows our expectations since the polymorphisms are within the disease gene. In family RB-32, an intragenic deletion in one copy of the RB gene, presumably causing the predisposition to the tumor, was identified by Southern blotting (data not shown). The deletion includes the region surrounding RB1.3, and hence carriers of the disease-predisposing allele are genotypically hemizygous for the A allele (A,-). Two unaffected members of pedigree RB-32

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are carriers for the disease-predisposition, based on analysis of RBl.3 (see Fig. 9). More happily, Fig. 9 shows that the other unaffected children in pedigree RB-32, as well as those in pedigrees RB-36 and RB-50, are not carriers of the cancer-predisposition and therefore will not pass the disease on to their children. These results highlight the diagnostic value of this class of human genetic markers that were heretofore unavailable for this purpose.

The data we present may also be used to estimate the level of heterozygosity in the human genome from a novel perspective. Previous estimates based on restriction enzyme screening may be subject to a bias because the sequences recognized by these enzymes do not necessarily reflect a random sampling. It is likely that a substantially higher level of polymorphism occurs at CpG pairs than elsewhere. This is reflected by the relatively high proportion of RFLPs revealed by such enzymes as Msp I (CCGG) and Taq I (TCGA). The method we describe is not subject to this bias. From the results of our screening, it can be calculated that genomic heterozygosity at this locus is approximately $h=0.00039$. If only the intron sequences are considered, this estimate increases to $h=0.00070$. These estimates are below the predictions of others, and may reflect the absence from our methods of the bias described above. However, an analogous calculation of heterozygosity ($0.00044 < h < 0.00087$) based instead on our

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initial RFLP screening is also below the estimates of others and is quite consistent with our estimate based on direct sequencing. It seems likely that the human retinoblastoma gene is intrinsically less polymorphic than many other regions of the genome. Although mutations in this gene are known to be early events in the formation of several types of cancer, it is unclear why polymorphism at this locus may have been selected against in human evolution.

The approach for detecting DSPs demonstrated here has several advantages over conventional RFLP-based screening. As we have argued, DSP screening by amplification and direct sequencing could increase by an order of magnitude the number of available polymorphic markers at any cloned locus. This technique encompasses and supercedes restriction enzyme-based screening since RFLPs and VNTRs may also be detected. As the only requirement for utilization of such markers is knowledge of a unique set of amplification primer sequences and of the polymorphism itself, publication of a polymorphism immediately makes it available to all readers. Hence, problems and delays associated with the physical transfer of plasmid DNAs between laboratories are avoided, and the costs of maintaining plasmid repositories will be ultimately reduced. In addition, rapid analysis of these polymorphic markers can be carried out on a large scale with the use of allele-specific oligonucleotide probes for direct hybridization to amplified DNA. Finally,

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based on our experience with both strategies at the same locus, we found the expense and effort required to locate DSPs by either method to be comparable.

Treatment of Patients Having a Defective Rb Gene

In addition to screening, the invention includes polypeptide therapy for those individuals determined to contain a defective Rb allele, and who therefore are at risk of developing retinoblastoma.

To prevent the formation of retinoblastoma in these individuals, the Rb polypeptide is administered therapeutically in an amount sufficient to inhibit retinoblastoma tumor formation or growth (anti-retinoblastoma-forming amount). An anti-retinoblastoma-forming dosage of the Rb polypeptide is 1 to 500 µg/kilogram of body weight/day. The Rb protein can be administered by injection with a pharmacologically acceptable carrier, either alone or in combination with another agent. Acceptable pharmacological carriers are those which dissolve the Rb polypeptide or hold it in suspension, and which are not toxic to the extent of permanently harming the patient. Preferred are aqueous solutions of salts or non-ionic compounds such as sodium chloride or glucose, most preferably at an isotonic concentration. Other agents may be present provided that they do not interfere with the action of the Rb polypeptide. Those skilled in the art will know, or will be able to ascertain with no more than routine experimentation,

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particular pharmacological carriers for this composition.

Rb polypeptide suitable for therapy can be prepared by any one of the following three conventional procedures. First, the Rb polypeptide can be produced by cloning the Rb cDNA from p4.7R into an appropriate mammalian expression vector, expressing the Rb gene product from this vector in an in vitro expression system, and isolating the Rb polypeptide from the medium or cells of the expression system. General expression vectors and systems are well known in the art.

Second, the Rb polypeptide can be produced using protein chemistry techniques, wherein the specific amino acid residues are joined together synthetically in the appropriate sequence.

Third, naturally occurring Rb protein can be isolated from total protein samples by affinity chromatography. Antibodies specific for the Rb protein are prepared by standard procedures (see below) and coupled to an inert matrix, which is then used to selectively bind the Rb proteins.

Immunodiagnosis of Retinoblastoma

This invention also includes methods for determining whether a particular tumor is the result of an Rb gene abnormality. Since osteosarcomas and certain undifferentiated tumors can result from detectable lesions in the Rb gene, immunodiagnosis can be used to aid in the diagnosis of such tumors.

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In order to produce anti-Rb antibody, a rabbit is immunized with either naturally occurring Rb protein or Rb polypeptide produced as described above. The anti-Rb antibody generated is then labeled, e.g., radioactively, fluorescently, or with an enzyme such as alkaline phosphatase. The labeled antibody is used to determine whether human tumors are of defective Rb gene origin. This can be carried out using any conventional technique. For example, the tumor sample can be liquified and tested against the labeled antibody using a conventional ELISA (Enzyme-linked immunosorbent assay) format. Alternatively, human tissue samples (e.g., biopsy samples) can be tested for expression of the retinoblastoma protein by other immunological techniques, see e.g., I. Roitt, Interaction of Antigen and Antibody, In Essential Immunology, Fifth edition, Boston: Blackwell Scientific Publications, 1984, pp. 145-75.

Immune complexes will be detected in tumor samples which have antigens (e.g., retinoblastoma polypeptide) reactive with anti-Rb antibody. Tumors which lack these antigens presumptively have a defect (e.g., mutation or a deletion) in the retinoblastoma gene.

Deposits

Plasmids p2AR3.8 and p2AR0.9 were deposited on July 17, 1987 with the American Type Culture Collection, Rockville, Maryland, and assigned ATCC accession numbers 40,241 and 40,242, respectively.

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The Applicants represent the ATCC is a depository affording permanence of the deposit and ready accessibility thereto by the public if a patent is granted. All restrictions on the availability to the public of the material so deposited will be irrevocably removed upon the granting of a patent. The material will be available during the pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 USC 122. The deposited material will be maintained with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposited microorganism, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of the patent, whichever period is longer. Applicant acknowledges its duty to replace the deposit should the depository be unable to furnish a sample when requested due to the condition of the deposit.

Other embodiments are within the following claims.

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Table 4. Polymorphic sequences characterized and primer pairs used for PCR-amplification

Polymorphic Sequence	Allele Frequency	Amplification Primer Pair	Fragment Size	Location
<u>RB1.2:</u> TAAATAAGAICTTAAG TAAATAAGA CTAAAG	>95% < 5%	5'-AAGTGTAATGTTTTTCTAAG-3' 5'-TAGCAGAGGTAAATTCCTC-3'	431 bp	124 bp from 5' end of exon 2
<u>RB1.3:</u> CAGAAATCGTTTCCTTT CAGAAATCAATTCCTTT	73% 27%	5'-TTCAATATATGCCATCAGA-3' 5'-GCTTACACATGAATAGTGAGAG-3'	530 bp	43 bp from 3' end of exon 3
<u>RB1.20:</u> GATTT(CIII) _n CCTTTT n=14-26	N/D	5'-AATTACAAGGTTGGTGG-3' 5'-CTTGTAATATGCCCTCAAT-3'	550 - 600 bp	54 bp from 3' end of exon 20
<u>RB1.26:</u> ATTTTIIAATCTGCAGT ATTTTIIAATCTGCAGT	85% 15%	5'-ATTCAGTGAAGATATCTAAT-3' 5'-TAGTTCCTCTTTGTAGTTCT-3'	683 bp	10 bp from 5' end of exon 26

Shown are the sequences and locations of the polymorphic sites and their immediate flanking regions. Also shown are the oligonucleotide primers used to amplify these sequences from human genomic DNA. 'Fragment size' refers to the PCR-amplified product. Allele frequencies are based on analysis of the following numbers of individuals (of mixed North American descent): (RB1.2)-15; (RB1.3)-41; (RB1.20)-14; (RB1.26)-27. Accurate allele frequencies for RB1.20 have not yet been determined, as numerous alleles were found.

Table 3

DNA sequence polymorphisms detected by direct sequencing

Base Pairs Screened		Polymorphisms
Introns	2072	4
Exons	1640	0
<hr/>		<hr/>
Totals	3712	4

DNA sequence polymorphisms found by direct sequencing of 13 separate PCR-amplified regions from the human retinoblastoma locus. DNA samples from a minimum of 9 unrelated individuals were examined for all bases screened. Bases that could not be scored unambiguously were excluded from this tabulation.

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Claims

1. Purified nucleic acid comprising a human retinoblastoma gene, or a fragment thereof comprising 15 or more bases, said nucleic acid being less than 100kb in size.
2. A vector comprising the nucleic acid of claim 1.
3. A cell transformed with DNA encoding retinoblastoma polypeptide or a fragment thereof.
4. The nucleic acid of claim 1, wherein said nucleic acid hybridizes specifically to said retinoblastoma gene under hybridizing conditions.
5. An isolated polypeptide encoded by the nucleic acid of claim 1.
6. An antibody produced to the polypeptide of claim 5.
7. An antibody produced to naturally occurring retinoblastoma polypeptide.
8. A method of detecting large deletions in the retinoblastoma gene of a human patient predisposing said patient to retinoblastoma, comprising the steps of:
hybridizing a nucleic acid sample from said patient with a probe specific for the retinoblastoma gene, and
determining the ability of said probe to hybridize to said nucleic acid,
wherein lack of hybridization to said nucleic acid indicates the presence of a large deletion in said gene.
9. A method of detecting large deletions in the retinoblastoma gene of a human patient that may predisposing said patient to retinoblastoma, comprising the steps of:

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generating nucleic acid fragments from a sample of said patient,

separating said fragments according to a determined physical property of said fragments,

hybridizing a probe specific for the retinoblastoma gene to said fragments,

detecting hybrids of said probe and said fragments, and

comparing said hybrids to hybrids detected from the hybridization of said probe and separated nucleic acid fragments from a normal retinoblastoma gene,

wherein the absence of hybrids, or the smaller size of said hybrids from the sample of said patient is an indication of large deletions in the retinoblastoma gene of said patient.

10. The method of claim 8 or 9, wherein the probe specific for the retinoblastoma gene is the cloned DNA in p4.7R, or a fragment thereof.

11. The method of claim 9, wherein the physical property is molecular weight.

12. A method of detecting small deletions or point mutations in the retinoblastoma gene of a human patient predisposing said patient to retinoblastoma, comprising the steps of:

determining the nucleotide sequence of a retinoblastoma allele, or subregion thereof, from said patient, and

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comparing said nucleotide sequence with the nucleotide sequence of a retinoblastoma allele or region thereof from a person not afflicted with retinoblastoma.

13. A method of detecting small deletions or point mutations in the retinoblastoma gene of a human patient predisposing said patient to retinoblastoma, comprising detecting mismatches between a nucleic acid sample from said patient and a detectable probe specific for the retinoblastoma gene from a person not afflicted with retinoblastoma, wherein mismatches are an indication of small deletions or mutations in the retinoblastoma gene of said patient.

14. A method of diagnosing predisposition of a human patient to retinoblastoma, comprising detecting the co-inheritance of retinoblastoma alleles of said patient with DNA polymorphisms in a pedigree analysis.

15. A method of detecting genetic polymorphisms, in the retinoblastoma gene of a human patient, that predispose said patient to retinoblastoma, comprising the steps of:

generating nucleic acid fragments from a sample of said patient,

separating said fragments according to a determined physical property of said fragments,

hybridizing a detectable nucleic acid probe capable of hybridizing to the wild type retinoblastoma gene to said fragments,

detecting hybrids of said probe and said fragments, and

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comparing said hybrids to hybrids detected from the hybridization of said probe and separated nucleic acid fragments from a sample of a parent of said patient,

wherein the co-inheritance of specific genetic polymorphisms with the retinoblastoma gene is an indication of the predisposition of said patient to retinoblastoma.

16. The method of claim 15, wherein said physical property is molecular weight.

17. A method of treating a human patient having a defective retinoblastoma gene comprising administering to said patient an anti-retinoblastoma-forming amount of the retinoblastoma polypeptide.

18. A composition suitable for treating a human patient having a defective retinoblastoma gene, comprising retinoblastoma polypeptide and a pharmacologically acceptable carrier therefor.

19. A method of detecting the presence, in a tumor sample, of a protein the absence of which is associated with a neoplasm, said method comprising producing an antibody to said protein, contacting said antibody with said tumor sample, and detecting immune complexes as an indication of the presence in said tumor sample of said protein.

20. A method of detecting the presence of the retinoblastoma protein in a tumor sample from a human patient, comprising the steps of:

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contacting said tumor sample with an antibody which specifically reacts with the retinoblastoma protein, and

determining whether immune complexes are formed with said antibody, the formation of said immune complexes being an indication that the tumor is not retinoblastoma and the absence of immune complexes indicating that the tumor is retinoblastoma.

21. The method of claim 20, wherein said antibody is a monoclonal antibody.

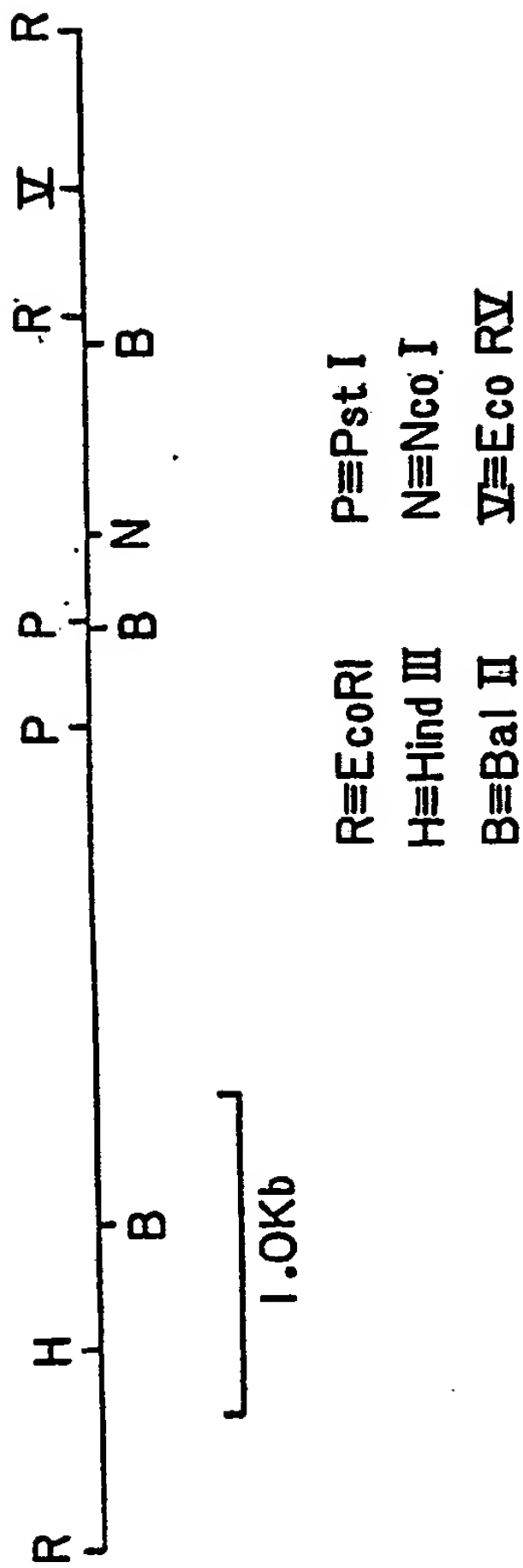
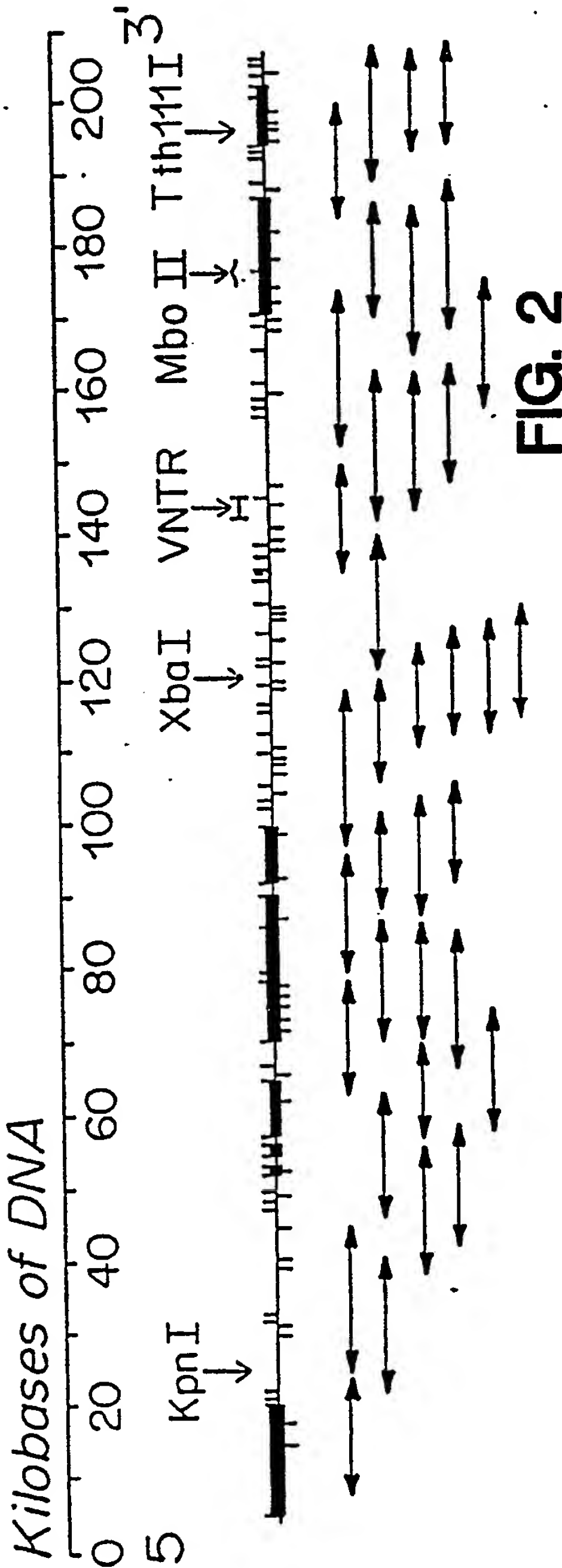


FIG. 1



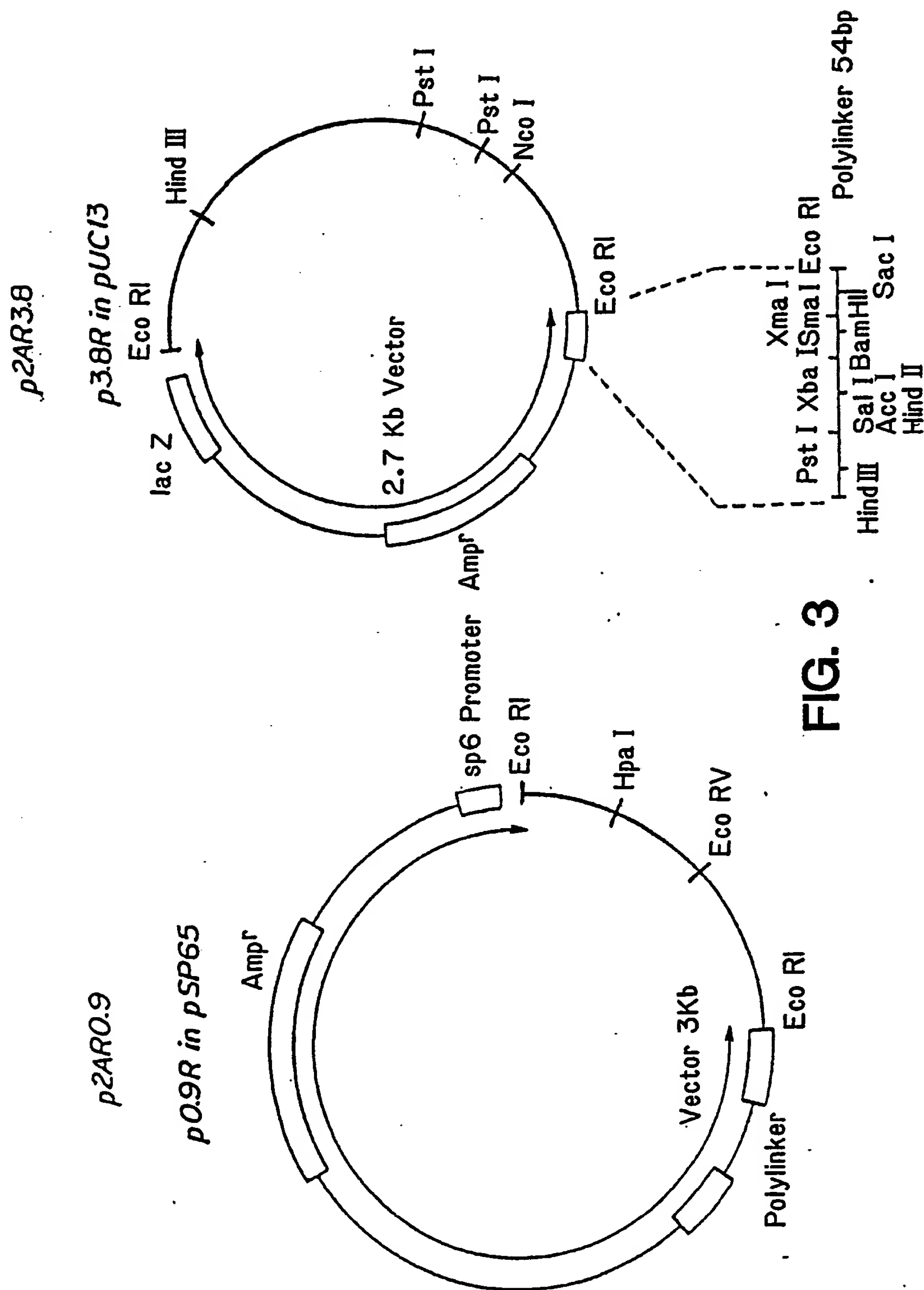
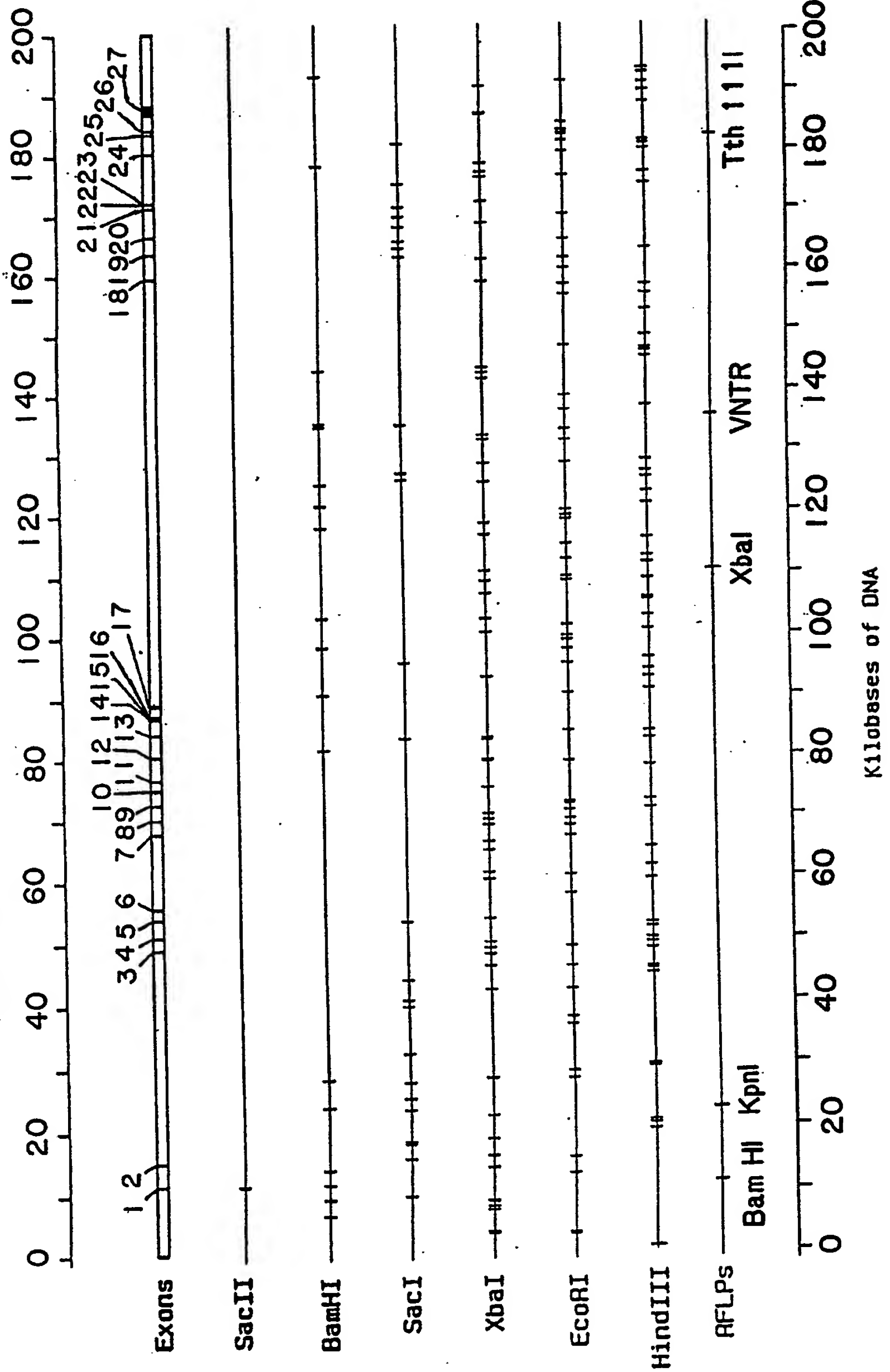


FIG. 3

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FIG. 4

MAP OF THE RETINOBLASTOMA GENE



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FIG. 5-1

GTCATGCCGCCCAAACCCCCGAAAAACGGCCGCCACCGCCGCCGCTGCCGCCGCGGAACCCCCGGCACC GGCCGCCGC...80
 CCCCTCCTGAGGAGGACCCAGAGCAGGACAGCGGCCCGGAGACCTGCCTCTCGTCAGGCTTGAGTTTGAAGAAACAGAAG...160
 AACCTGATTTTACTGCATTATGTCAGAAATTAAGATACCAGATCATGTCAGAGAGAGAGCTTGGTTAACTTGGGAGAAA...240
 GTTTCATCTGTGGATGGAGTATTGGGAGGTTATATTCAAAGAAAAAGGAAGTGTGGGAATCTGTATCTTTATTGCAGC...320
 AGTTGACCTAGATGAG.....336

337	ATG TCG TTC ACT TTT ACT GAG CTA CAG AAA AAC ATA GAA ATC	378
	M S F T F T E L Q K N I E I	
379	AGT GTC CAT AAA TTC TTT AAC TTA CTA AAA GAA ATT GAT ACC	420
	S V H K F F N L L K E I D T	
421	AGT ACC AAA GTT GAT AAT GCT ATG TCA AGA CTG TTG AAG AAG	462
	S T K V D N A M S R L L K K	
463	TAT GAT GTA TTG TTT GCA CTC TTC AGC AAA TTG GAA AGG ACA	504
	Y D V L F A L F S K L E R T	
505	TGT GAA CTT ATA TAT TTG ACA CAA CCC AGC AGT TCG ATA TCT	546
	C E L I Y L T Q P S S S I S	
547	ACT GAA ATA AAT TCT GCA TTG GTG CTA AAA GTT TCT TGG ATC	588
	T E I N S A L V L K V S W I	
589	ACA TTT TTA TTA GCT AAA GGG GAA GTA TTA CAA ATG GAA GAT	630
	T F L L A K G E V L Q M E D	
631	GAT CTG GTG ATT TCA TTT CAG TTA ATG CTA TGT GTC CTT GAC	672
	D L V I S F Q L M L C V L D	
673	TAT TTT ATT AAA CTC TCA CCT CCC ATG TTG CTC AAA GAA CCA	714
	Y F I K L S P P M L L K E P	
715	TAT AAA ACA GCT GTT ATA CCC ATT AAT GGT TCA CCT CGA ACA	756
	Y K T A V I P I N G S P R T	
757	CCC AGG CGA GGT CAG AAC AGG AGT GCA CGG ATA GCA AAA CAA	798
	P R R G Q N R S A R I A K Q	
799	CTA GAA AAT GAT ACA AGA ATT ATT GAA GTT CTC TGT AAA GAA	840
	L E N D T R I I E V L C K E	
841	CAT GAA TGT AAT ATA GAT GAG GTG AAA AAT GTT TAT TTC AAA	882
	H E C N I D E V K N V Y F K	
883	AAT TTT ATA CCT TTT ATG AAT TCT CTT GGA CTT GTA ACA TCT	924
	N F I P F M N S L G L V T S	
925	AAT GGA CTT CCA GAG GTT GAA AAT CTT TCT AAA CGA TAC GAA	966
	N G L P E V E N L S K R Y E	
967	GAA ATT TAT CTT AAA AAT AAA GAT CTA GAT GCA AGA TTA TTT	1008
	E I Y L K N K D L D A R L F	
1009	TTG GAT CAT GAT AAA ACT CTT CAG ACT GAT TCT ATA GAC AGT	1050
	L D H D K T L Q T D S I D S	
1051	TTT GAA ACA CAG AGA ACA CCA CGA AAA AGT AAC CTT GAT GAA	1092
	F E T Q R T P R K S N L D E	
1093	GAG GTG AAT GTA ATT CCT CCA CAC ACT CCA GTT AGG ACT GTT	1134
	E V N V I P P H T P V R T V	
1135	ATG AAC ACT ATC CAA CAA TTA ATG ATG ATT TTA AAT TCA GCA	1176
	H N T I Q Q L M M I L N S A	
1177	AGT GAT CAA CCT TCA GAA AAT CTG ATT TCC TAT TTT AAC AAC	1218
	S D Q P S E N L I S Y F N N	

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FIG. 5-2

1219	TGC ACA GTG AAT CCA AAA GAA AGT ATA CTG AAA AGA GTG AAG	1260
	C T V N P K E S I L K R V K	
1261	GAT ATA GGA TAC ATC TTT AAA GAG AAA TTT GCT AAA GCT GTG	1302
	D I G Y I F K E K F A K A V	
1303	GGA CAG GGT TGT GTC GAA ATT GGA TCA CAG CGA TAC AAA CTT	1344
	G Q G C V E I G S Q R Y K L	
1345	GGA GTT CGC TTG TAT TAC CGA GTA ATG GAA TCC ATG CTT AAA	1386
	G V R L Y Y R V H E S M L K	
1387	TCA GAA GAA GAA CGA TTA TCC ATT CAA AAT TTT AGC AAA CTT	1428
	S E E E R L S I Q N F S K L	
1429	CTG AAT GAC AAC ATT TTT CAT ATG TCT TTA TTG GCG TGC GCT	1470
	L N D N I F H M S L L A C A	
1471	CTT GAG GTT GTA ATG GCC ACA TAT AGC AGA AGT ACA TCT CAG	1512
	L E V V H A T Y S R S T S Q	
1513	AAT CTT GAT TCT GGA ACA GAT TTG TCT TTC CCA TGG ATT CTG	1554
	N L D S G T D L S F P W I L	
1555	AAT GTG CTT AAT TTA AAA GCC TTT GAT TTT TAC AAA GTG ATC	1596
	N V L N L K A F D F Y K V I	
1597	GAA AGT TTT ATC AAA GCA GAA GGC AAC TTG ACA AGA GAA ATG	1638
	E S F I K A E G N L T R E M	
1639	ATA AAA CAT TTA GAA CGA TGT GAA CAT CGA ATC ATG GAA TCC	1680
	I K H L E R L E H R I M E S	
1681	CTT GCA TGG CTC TCA GAT TCA CCT TTA TTT GAT CTT ATT AAA	1722
	L A W L S D S P L F D L I K	
1723	CAA TCA AAG GAC CGA GAA GGA CCA ACT GAT CAC CTT GAA TCT	1764
	Q S K D R E G P T D H L E S	
1765	GCT TGT CCT CTT AAT CTT CCT CTC CAG AAT AAT CAC ACT GCA	1806
	A C P L N L P L Q N N H T A	
1807	GCA GAT ATG TAT CTT TCT CCT GTA AGA TCT CCA AAG AAA AAA	1848
	A D M Y L S P V R S P K K K	
1849	GGT TCA ACT ACG CGT GTA AAT TCT ACT GCA AAT GCA GAG ACA	1890
	G S T T R V N S T A N A E T	
1891	CAA GCA ACC TCA GCC TTC CAG ACC CAG AAG CCA TTG AAA TCT	1932
	Q A T S A F Q T Q K P L K S	
1933	ACC TCT CTT TCA CTG TTT TAT AAA AAA GTG TAT CGG CTA GCC	1974
	T S L S L F Y K K V Y R L A	
1975	TAT CTC CGG CTA AAT ACA CTT TGT GAA CGC CTT CTG TCT GAG	2016
	Y L R L N T L C E R L L S E	
2017	CAC CCA GAA TTA GAA CAT ATC ATC TGG ACC CTT TTC CAG CAC	2058
	H P E L E H I I W T L F Q H	
2059	ACC CTG CAG AAT GAG TAT GAA CTC ATG AGA GAC AGG CAT TTG	2100
	T L Q N E Y E L M R D R H L	
2101	GAC CAA ATT ATG ATG TGT TCC ATG TAT GGC ATA TGC AAA GTG	2142
	D Q I M M C S H Y G I C K V	
2143	AAG AAT ATA GAC CTT AAA TTC AAA ATC ATT GTA ACA GCA TAC	2184
	K N I D L K F K I I V T A Y	

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FIG. 5-3

2185	AAG GAT CTT CCT CAT GCT GTT CAG GAG ACA TTC AAA CGT GTT	2226
	K D L P H A V Q E T F K R V	
2227	TTG ATC AAA GAA GAG GAG TAT GAT TCT ATT ATA GTA TTC TAT	2268
	L I K E E E Y D S I I V F Y	
2269	AAC TCG GTC TTC ATG CAG AGA CTG AAA ACA AAT ATT TTG CAG	2310
	N S V F H Q R L K T N I L Q	
2311	TAT GCT TCC ACC AGG CCC CCT ACC TTG TCA CCA ATA CCT CAC	2352
	Y A S T R P P T L S P I P H	
2353	ATT CCT CGA AGC CCT TAC AAG TTT CCT AGT TCA CCC TTA CGG	2394
	I P R S P Y K F P S S P L R	
2395	ATT CCT GGA GGG AAC ATC TAT ATT TCA CCC CTG AAG AGT CCA	2436
	I P G G N I Y I S P L K S P	
2437	TAT AAA ATT TCA GAA GGT CTG CCA ACA CCA ACA AAA ATG ACT	2478
	Y K I S E G L P T P T K M T	
2479	CCA AGA TCA AGA ATC TTA GTA TCA ATT GGT GAA TCA TTC GGG	2520
	P R S R I L V S I G E S F G	
2521	ACT TCT GAG AAG TTC CAG AAA ATA AAT CAG ATG GTA TGT AAC	2562
	T S E K F Q K I N Q M V C N	
2563	AGC GAC CGT GTG CTC AAA AGA AGT GCT GAA GGA AGC AAC CCT	2604
	S D R V L K R S A E G S N P	
2605	CCT AAA CCA CTG AAA AAA CTA CGC TTT CAT ATT GAA GGA TCA	2646
	P K P L K K L R F D I E G S	
2647	GAT GAA GCA GAT GGA AGT AAA CAT CTC CCA GGA GAG TCC AAA	2688
	D E A D G S K H L P G E S K	
2689	TTT CAG CAG AAA CTG GCA GAA ATG ACT TCT ACT CGA ACA CGA	2730
	F Q Q K L A E M T S T R T R	
2731	ATG CAA AAG CAG AAA ATG AAT GAT AGC ATG GAT ACC TCA AAC	2772
	M Q K Q K M N D S M D T S N	
2773	AAG GAA GAG AAA	2784
	K E E K	

TGAGGATCTCAGGACCTTGGTGGACACTGTGTACACCTCTGGATTCAATTGTCTCTCACAGATGTGACTGTATAACTTTCC 2864
CAGGTTCTGTTTATGGCCACATTTAATATCTTCAGCTCTTTTGTGGATATAAAATGTGCAGATGCAATTGTTTGGGTGA 2944
TTCCTAAGCCACTTGAAATGTTAGTCATTGTTATTTATACAAGATTGAAAATCTTGTGTAAATCCTGCCATTTAAAAAGT 3024
TGTAGCAGATTGTTTCTCTTCCAAAGTAAATTTGCTGTGCTTTATGGATAGTAAGAATGGCCCTAGAGTGGGAGTCCTG 3104
ATAACCCAGGCCTGTCTGACTACTTTGCCTTCTTTGTAGCATATAGGTGATGTTTGTCTTGTGTTTTATTAATTTATAT 3184
GTATATTTTTTTAATTTAACATGAACACCCTTAGAAAAATGTGTCTATCTATCTTCCAAATGCAATTTGATTGACTGCCC 3264
ATTCACCAAAATTATCCTGAACTCTTCTGCAAAAATGGATATTATTAGAAATTAGAAAAAAATTAATAATTTTACACATT 3344
AGATTTTATTTTACTATTGGAATCTGATATACTGTGTGCTTGTGTTTTATAAAATTTTGTCTTTAATTAATAAAAGCTGGA 3424
AGCAAAGTATAACCATATGATACTATCATACTACTGAAACAGATTTACATCCTCAGAATGTAAAAGAACTTACTGATTAT 3504
TTTCTTCATCCAACCTTATGTTTTTAAATGAGGATTATTGATAGTACTCTTGGTTTTTATACCATTGAGATCACTGAATTT 3584
ATAAAGTACCCATCTAGTACTTGAAAAAGTAAAGTGTTCTGCCAGATCTTAGGTATAGAGGACCCTAACACAGTATATCC 3664
CAAGTGCACTTTCTAATGTTTCTGGGTCCTGAAGAAATTAAGATACAAATTAATTTTACTCCATAAACAGACTGTAAATTA 3744
TAGGAGCCTTAATTTTTTTTTTTCATAGAGATTTGTCTAATTGCATCTCAAAATTAATCTGCCCTCCTTAATTTGGGAAGGT 3824
TTGTGTTTTCTCTGGAATGGTACATGTCTTCCATGTATCTTTTGAAGTGGCAATTGTCTATTTATCTTTTATTTTTTAA 3904
GTCAGTATGGTCTAACACTGGCATGTTCAAAGCCACATTATTTCTAGTCCAAAATTACAAGTAATCAAGGGTCATTATGG 3984
GTTAGGCATTAATGTTTCTATCTGATTTTGTGCAAAAGCTTCAAAATTAACAGCTGCATTAGAAAAAGAGGCGCTTCTC 4064
CCCTCCCCTACACCTAAAGGTGATTAAACTATCTTGTGTGATTAACCTATTTAGAGATGCTGTAACCTTAAATAGGGG 4144
ATATTTAAGGTAGCTTCAGCTAGCTTTTAGGAAAACTACTTTGTCTAACTCAGAATTATTTTTAAAAAGAAATCTGGTCT 4224
TGTTAGAAAAACAAAATTTTATTTTGTGCTCATTTAAGTTTCAAACTTACTATTTTGACAGTTATTTTGATAACAATGACA 4304
CTAGAAAACTTGACTCCATTTTCATCATTGTTTCTGCATGAATATCATACAATCAGTTAGTTTTTAGGTCAAGGGCTTAC 4384
TATTTCTGGGTCTTTTGTCTACTAAGTTCACATTAGAATTAGTGCCAGAATTTTAGGAACTTCAGAGATCGTGTATTGAGA 4464
TTTCTTAAATAATGCTTCAGATATTATTGCTTTATTGCTTTTTTGTATTGGTTAAAACTGTACATTTAAATTTGCTATGT 4544
TACTATTTTCTACAATTAATAGTTTGTCTATTTTAAATAAATTAGTTGTTAG..... 4597

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FIG. 6-1

	aaggagggag	agtggngtcc	ngnngagggt	gcactagcca	gatattctgc	ggggcccgag	060
	agtcttccct	atcagacccc	gggatagggg	tgaggCCCAC	AGTCACCCAC	CAGACTCTTT	120
	GTATAGCCCC	GTTAAGTGCA	CCCCGGCCTG	GAGGGGGTGG	TTCTGGGTAG	AAGCACGTCC	180
	GGGCCGCGCC	GGATGCCTCC	TGGAAGGCGC	CTGGACCCAC	GCCAGGTTTC	CCAGTTTAAT	240
	TCCTCATGAC	TTAGNGTCCC	AGCCNGCGCA	CCGACCAGCG	CCCCAGTTCC	CCACAGACGC	300
	CGGCGNGNNC	GGGAGCCTGC	GGACGTGAGC	GCGGGCGGAA	GTGACGTTTT	CCGCGGTTGG	360
<u>SacII</u>	ACGCGGCGCT	CAGTTGCCGG	GCGGGGGAGG	GCGCGTCCGG	TTTTTCTCAG	GGGACGTTGA	420
EXON 1	start of cDNA sequence						
	AATTATTTTT	GTAACGGGAG	TCGGGAGAGG	ACGGGGCGTG	CCCCGCGTGC	GCGGCGGTCC	480
	TCCTCCCCGG	CGCTCCTCCA	CAGCTCGCTG	GCTCCCCGCC	CGGAAAGGCG	TCATGCCGCC	540
					MetProPr	(3)	
	CAAAACCCCC	CGAAAAACGG	CCGCCACCGC	CGCCGCTGCC	GCCGCGGAAC	CCCCGGCACC	600
<u>SacII</u>	oLysThrPro	ArgLysThrA	laAlaThrAl	aAlaAlaAla	AlaAlaGluP	roProAlaPr	(23)
	GCCGCCCCCG	CCCCCTCCTG	AGGAGGACCC	AGAGCAGGAC	AGCGGCCCGG	AGGACCTGCC	660
	oProProPro	ProProProG	luGluAspPr	oGluGlnAsp	SerGlyProG	luAspLeuPr	(43)
	TCTCGTCAGG	TGAGCGAGCA	GAGCGCGTCN	CTCACGCGGG	AAGGGCGCCC	CGGGTGTGCG	720
	oLeuValAr						(46)
	TAGGGCGGGC	GCAAGGCGgC	TCGGCGGGGA	CCCGTCCTCG	CCAGGGgCCG	GGTCCcgGNG	780
	GGAGGAGGCG	CCCTCCCTGC	CCCCCGCCAC	GGcggaGCGT	CTGCAGAATG	GTGACAGGAT	840
	TCTGGGTTCT	TGGGCGAGGG	GTCTCGGCTT	CAACTTGACA	GGTGTGCGGC	GGGTggggct	900
	agnntcctga	gagaagtga	aggtgcagtt	ccctcttgtg	agnctcggan	ncagaggntc	960
	gttgcgagcg	tncatcagac	aaaaaatga	aaaataaaaa	tacaaaaa		1008
	--2.9 kb--						
	cccaaacagc	tttagctatt	acatttactt	tccttcacag	aagtgttttg	ctgctttgaa	060
	gatatttgac	ttaccatgca	agcaaatatt	tttcaactgtg	tggtatcctt	attttggaat	120
	gaccatgaaa	aagataatca	tatgnnnaaa	tttgaagtgt	aatgtttttc	taagataaaa	180
	taagatctta	AAGTATTTAA	TAATGTTCTT	TTTCACAGTA	GTGTTATGTG	CAAACCTATTG	240
	AAACAAGTAT	GTAAGTGAATC	AATTTGATTT	ATAAGATATG	CCAATTATAT	GATTATTTTC	300
EXON 2	ATTTGGTAGG	CTTGAGTTTG	AAGAAACAGA	AGAACCTGAT	TTTACTGCAT	TATGTCAGAA	360
	g	LeuGluPheG	luGluThrGl	uGluProAsp	PheActAlaL	euCysGlnLy	(63)
	ATTAAAGATA	CCAGATCATG	TCAGAGAGAG	AGCTTGGTTA	ACTTGGGAGA	AAGTTTCATC	420
	sLeuLysIle	ProAspHisV	alArgGluAr	gAleTrpLeu	ThrTrpGluL	ysValSerSe	(83)
	TGTGGATGGA	GTATTGGTAA	GGATTTTCTT	AAAACGTTTT	GAAATTTTTT	TTTCTCATTT	480
	rValAspGly	ValLeu					(88)
	TAAAACCAAC	TTCAAATCAC	TATACAAAAA	TTGAAAGATA	GAAAAATATA	AAGACAATAA	540
	AAGctaataa	taattccatt	acccagagga	aatttacctc	tgctaacatt	aaaaatgttt	600
	gaggccgggc	acgtgggttc	tgccgtgaat	cctaccactt	tgggaggetg	aggcaggtgg	660
<u>SacI</u>	attgcctgag	ctcaggaggt	cgagaccagc	ctgggcaaca	tggt		704
	--33 kb--						
	ctattttgaga	tgactgaccc	ctaaagttec	acaataacta	tttaattttt	tatctttcta	060
	atactttttt	gccttataat	ataaaatttg	aatgtttgtt	attagtgtga	aatgaaatcc	120
	tttcaaatat	atgccatcag	aaggatgtgt	tacaaatata	cagtATTACA	AACATTATT	180
	TTGTATGCTG	AATAAGAAAA	AATCAGTTAT	AATACAGTTT	TAACATAGTA	TCCAGTGTGT	240
EXON 3	GAATTATTTA	ATGAAATATT	TGATCTTTAT	TTTTTGTTCC	AGGGAGGTTA	TATTCAAAAG	300
					GlyGlyTy	rIleGlnLys	(94)
	AAAAAGGAAC	TGTGGGGAAT	CTGTATCTTT	ATTGCAGCAG	TTGACCTAGA	TGAGATGTCC	360
	LysLysGluL	euTrpGlyIl	eCysIlePhe	IleAlaAlaV	alAspLeuAs	pGluMetSer	(114)
	TTCACTTTTA	CTGAGCTACA	GAAAAACATA	GAAATCAGGT	AAAGTTTCTT	GTATAAATAT	420
	PheThrPheT	hrGluLeuGl	nLysAsnIle	GluIleSe			(127)
<u>EcoRI</u>	AAGCCTCTGC	CATAAAAGGA	AACGAATTCT	GGATTTTCCT	CTCAATAGAC	TTTTGTGAAT	480
	TAGTGAGAAA	TGCTAAAATA	AAGTAAAACA	AAAAGAACTT	GGACCAAATA	GTGAACTGCC	540
	ATTCTCTCAT	GGAGCCGTTA	TGAAAGTGTA	TTTATGCTGT	ATTTCTTTAA	GAGGTAGCAG	600
	TTTGTGTCCT	GGAAAAATTT	TCATTGTGTC	TCTCACTATT	CATGTGTAAAG	C	651
	--1.6 kb--						

	gcataaggatat	atagataata	gaggtgtaag	ttgaaggcta	attattttttg	caaaaagtaa	060
	ttccttccaa	aggatatagt	agtgatttga	tgtagagctg	ataatcTTTT	GAATTGAAAT	120
	ATCTATGATT	TGAAAACGAA	ATAACACAAA	TTTTTAAGGT	TACTGATTTA	CTTTTTTCTA	180
EXON 4	TTCTTTCCTT	TGTAGTGTCC	ATAAATTCTT	TAACCTACTA	AAAGAAATTG	ATACCAGTAC	240
		rValH	isLysPhePh	eAsnLeuLeu	LysGluIleA	spThrSerTh	(142)
	CAAAGTTGAT	AATGCTATGT	CAAGACTGTT	GAAGAAGTAT	GATGTATTGT	TTGCACTCTT	300
	rLysValAsp	AsnAlaMetS	erArgLeuLe	uLysLysTyr	AspValLeuP	heAlaLeuPh	(162)
	CAGCAAATTG	GAAAGGTAAA	GTAAACATTT	TATTAGGGTT	ACACTCTGAT	TTTTTATGTC	360
	eSerLysLeu	GluAr					(167)
	ATTGTTTACA	ATTAGATTCT	GGGAATTATT	TAACACATTT	AGTAAAGTTA	GTAAGTATTA	420
	ATTCTTAGac	ttgtcccttt	taatgtttagc	tcattaatctc	ttagctttct	tatttatcca	480
	gtaatatgca	ttctgaatgc	ttcctggaaa	attaaccgtt	attatccttt	catgtctcca	540
	tttgttttca	aaacttagct	tatcgagtat				570
			--2.1 kb--				
	gagatatatta	aagagnaact	ttactaacct	taggtggatc	agctgggtgt	tttctatctt	060
	atttataacct	ttttttttgaa	GACTAATTGA	GAGGATTAAC	TGTAATTATA	TATTAAAGTG	120
	ATGTGAGATG	TCATAAATTG	GGAAAATCTA	CTTGAACCTT	GTTTTATAAT	GCTATATATT	180
	TTTTGTTTTT	AAAATATATA	CTTCTTAAAA	GAAGATGAAT	AAAGCATGAG	AAACTACTA	240
EXON 5	TGACTTCTAA	ATTACGAAAA	AATGTAAAAA	AGTCATAATG	TTTTTCTTTT	CAGGACATGT	300
					gThrCys		(169)
	GAACCTATAT	ATTTGACACA	ACCCAGCAGT	TCGTAAGTAG	TTCACAGAAT	GTTATTTTTC	360
	GluLeuIleT	yrLeuThrGl	nProSerSer	Se			(180)
	ACTTAAAAAA	AAAGATTTTT	ATGGAATAAT	CTCAAACATC	TTGATAGTTA	GGGTTAGTTT	420
	GATCGATTAT	AGCAGGCTAC	Ttcataaatt	aagcccatag	atttaagtcc	tgtgtagatt	480
	atttatcttc	tcacaaagaa	aatagtataa	aatacatgcc	ttgtactaca	aagaagaact	540
	aataaggtgg	aattgattca	ggacagcata	tcaccaactc	tgagaaaaat	gcaacaaatg	600
	caaattcatt	gactaa					616
			--1.4 kb--				
	aaatggactg	cattctatta	tgcatttaac	taaggtcatt	tttttttttaa	tGCACAAAAA	060
	GAAACACCCA	AAAGATATAT	CTGGAAACT	TTCTTTCAGT	GATACATTTT	TCCTGTTTTT	120
EXON 6	TTTCTGCTTT	CTATTTGTTT	AATAGGATAT	CTACTGAAAT	AAATTCTGCA	TTGGTGCTAA	180
		rIleS	erThrGluIl	eAsnSerAla	LeuValLeuL		(192)
	AAGTTTCTTG	GATCACATTT	TTATTAGCTA	AAGGTAAGTT	CATTATATTT	ATTAAATGCT	240
	ysValSerTr	pIleThrPhe	LeuLeuAlaL	ysG			(203)
	AATATTTCAA	ATGTAATAAT	TAAATTGGCA	TTCCTTTGGA	CTAAATTCCC	CAATTTTTTAT	300
	TGAGTAATGT	ACTCCTccct	cattctctgc	ttggcttatt	aactgttagc	aagttccctat	360
	aattctggta	ctagaascaa	ccttggaat	gctttattta	atntttggtt	ctaatattec	420
	atcttccttc	cctt					434
			--11.5 kb--				
	tttatagtga	ttttagacat	aaagaattaa	ttataacaga	aatagcttaa	atgtaaaatt	060
	ctcagagtag	agcttaacac	ttgatttata	attccataac	tttacatatt	tCTATTTTAC	120
	ATATTTTATA	CCTTTTAAAA	CAGATTTTTT	TTTTTTTTTAC	AAAAAAAAGA	AAGAAAATCT	180
	TTACCATGCT	GATAGTGATT	GTTGAATGAA	TAAATTTATG	GATATACTCT	ACCCTGCGAT	240
	TTTCTCTCAT	ACAAAGATCT	GAATCTCTAA	CTTCTTTTAA	AAATGTACAT	TTTTTTTTTCA	300
EXON 7	GGGGAAGTAT	TACAAATGGA	AGATGATCTG	GTGATTTTAT	TTCAGTTAAT	GCTATGTGTC	360
	lyGluValL	euGlnMetGl	uAspAspLeu	ValIleSerP	heGlnLeuMe	tLeuCysVal	(222)
	CTTGACTATT	TTATTAAACT	CTCACCTCCC	ATGTTGCTCA	AAGAACCATA	TAGTAAGTAT	420
	LeuAspTyrP	heIleLysLe	uSerProPro	MetLeuLeuL	ysGluProTy	rL	(240)
	TTAATTTATG	CCCCTTTTAC	TTTCTCATTC	AGCAGTTGCT	TATTGAATGT	CTAGTGGGTA	480
	CCAAACATGG	TTCTAAGGCT	GACAGGATGA	TAAAAAATAA	ATCagacatg	gactttgccc	540
	ataagtagtg	taagttatag	aaggaaagat	aagacatgga	aacaaatgat	tagagtatat	600
	ggtagaaagt	ggtttctgggt	caaaatacaa	caaatggagg	tttggggagac	aagaag	656
			--1.8 kb--				

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FIG. 6-3

	gctattecacat	gccttctctt	tgtatttggt	tatgagactg	tagtttacag	ttcttttttg	060
	gannagagta	gaagagggat	gCAAAACTA	ATATTAGTAC	ATAATTTGTA	GTAGATATGG	120
	ATGAAATTGT	TATCCTTCTA	ATGAAACCTA	ATAAGTAAAA	GTAGTAGAAT	GTTACCAAGA	180
	TTATTTTGA	CCTAAGTAT	AGTTAGAATA	CTTCATTATT	TTATATGATG	GATGTACAAT	240
EXON 8	TGTTCTTATC	TAATTTACCA	CTTTTACAGA	AACAGCTGTT	ATACCCATTA	ATGGTTCACC	300
			y	sThrAlaVal	IleProIleA	snGlySerPr	(250)
	TCGAACACCC	AGGCGAGGTC	AGAACAGGAG	TGCACGGATA	GCAAAACAAC	TAGAAAATGA	360
	oArgThrPro	ArgArgGlyG	lnAsnArgSe	rAlaArgIle	AlaLysGlnL	euGluAsnAs	(270)
	TACAAGAATT	ATTGAAGTTC	TCTGTAAAGA	ACATGAATGT	AATATAGATG	AGGTAATTTA	420
	pThrArgIle	IleGluValL	euCysLysG1	uHisGluCys	AsnIleAspG	lu	(287)
	ACTTCATGAT	TTCTTTAAAA	CAGTTAAAGT	AGATTTAGAT	GTAAGTTCTC	CCTAACAATA	480
	TTTACTTCTT	TTGTTATGAG	CATGTTTTTT	TTGTAATTAG	TGCTAACTCT	TTTGCAGTAG	540
	CAAAATATTT	AGAAAAatta	attcggtata	tttagttact	ttgatttaag	agagtagctc	600
	cctcactct						609
	--1.8 kb--						
	aagcattgaa	gctgtaatgc	atgtgattgc	acctgtgaat	agccactaca	cttcagccta	060
	ggcaatatag	agagaccctt	tctcTAAGAA	AATAATAAAA	AATAAAAAAG	TTATACACAG	120
	ATTTTTTACT	GCATGGGGGA	TTGACACCTC	TAACCTTACCC	TGCATTGTTC	AAGAGTCAAG	180
EXON 9	AGATTAGATT	TTGTTTTAAA	TTTTAATGAT	CATGTTGTAA	CTTCATCTTT	TTCAGGTGAA	240
					VallY		(289)
<u>EcoRI</u>	AAATGTTTAT	TTCAAAAATT	TTATACCTTT	TATGAATTCT	CTTGGACTTG	TAACATCTAA	300
	sAsnValTyr	PheLysAsnP	heIleProPh	eMetAsnSer	LeuGlyLeuV	alThrSerAs	(309)
	TGGACTTCCA	GAGGTAATCT	GAAAGGAAAT	TTAATAAAAT	ATTAATGTTT	TGAGACTGTG	360
	nGlyLeuPro	Glu					(313)
	GAGGGAGGAT	AATTGTCTAA	CTTCTTAGA	TCAATTTACT	GTGTATCACA	TTTTTTTTTT	420
	GCCCAAGAAG	AATCTAGCCA	AGTAGAATTG	TGGTGAAACT	AACTTTTGTA	TAGTAacaaa	480
							486
<u>HindIII</u>	aagctt						
	--1.9 kb--						
	gtagcattgg	ctatctttgt	ctacataaaa	ttctaataaa	tattttctat	gcacgAAATA	060
	GACCTAAAAT	CAAAGTTGAA	CAAATGTTGC	AATTTTCTGT	ACCTCACTTT	TAGATAGACC	120
	TTATTTATAT	TGCATGCGAA	CTCAGTGTAT	ATTACAAAAT	TAAATGTATA	TTATACAAAA	180
	ATTCTTTAAT	GAAATCTGTG	CCTCTGTGTG	CTGAGAGATG	TAATGACATG	TAAAGGATAA	240
EXON 10	TTGTCAGTGA	CTTTTTTCTT	TCAAGGTTGA	AAATCTTTCT	AAACGATACG	AAGAAATTTA	300
			ValG1	uAsnLeuSer	LysArgTyrG	luGluIleTy	(325)
<u>XbaI</u>	TCTTAAAAAT	AAAGATCTAG	ATGCAAGATT	ATTTTGGAT	CATGATAAAA	CTCTTCAGAC	360
	rLeuLysAsn	LysAspLeuA	spAlaArgLe	uPheLeuAsp	HisAspLysT	hrLeuGlnTh	(345)
	TGATTCTATA	GACAGGTATT	GCACATGGTA	TATTTGATTG	ATTTGCTTTA	GATATAGGTT	420
	rAspSerIle	AspSe					(350)
	GATACTGATA	TAGGTAGATT	ATATAGTCTT	TAGCTTAGTG	ACCTTTAGAT	ATCATTTATA	480
	ACAAATTACT	TTCAAATGTC	TTTATACAAA	GAAAAGTTTA	ACAGTATTTT	AAGcatataa	540
	cttatctaca	aatanagatt	taatgtgaat	tgtgtgtcct	ataacagtta	ccttttttnca	600
	gttaactgaa	tataatTTTT	aaaatgtgca	ccaaagata	atggcta		647
	--1.0 kb--						

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FIG. 6-4

	aataactgaac	aacttggtta	tcaataaccnc	cagggagaag	catctgactt	tcacttttta	060
	aaaaagactt	aatgattggt	atacctcttt	gtcataaaca	taatggaaag	agacccacaa	120
	ttaaaaagng	tagtgAAAGG	TATTTTATTT	AAGCAGCAGC	TGGGTCATCT	ATTTTCTATC	180
	CTATCTATTA	TTGAGTTATC	ATTTTATATG	ATTTTATGAG	ACAACAGAAG	CATTATACTG	240
	CTTTTTTGAT	GCATAAAGCA	CAAATTGTAA	ATTTTCAGTA	TGTGAATGAC	TTCAC TTATT	300
EXON 11	GTTATTTAGT	TTTGAAACAC	AGAGAACACC	ACGAAAAAGT	AACCTTGATG	AAGAGGTGAA	360
		r PheGluThrG	lnArgThrPr	oArgLysSer	AsnLeuAspG	luGluValAs	(367)
	TGTAATTCCT	CCACACACTC	CAGTTAGGTA	TGAATTTTCC	TACTTTTAAT	TATATTATAA	420
	nValIlePro	ProHisThrP	roValAr				(326)
	TTTTGTTATT	CATGGCTTTA	TAGTGTTC	GATTGTTC	CGTTTCTTTA	TGTATTCATA	480
	CATACATGTA	AGAAATATAT	ATTGAAGGCC	AGGTGTGGTG	GATCACACCT	GTAATCCCAG	540
	CACTTTGGGA	GGCCAAGGCG	GGCAGATCAC	CTGAGGTTAG	GAGTTTGaga	ccggcctggc	600
	caacatggtg	aaaccccgtc	tctactagaa	atacaaaaat	tagctggggg	tgggtggtgtg	660
	tgccctgtaat	ccagctgctc					680
	--3.2 kb--						
	caataccatt	ttgttgccag	ttatatagtt	ctcctaasaa	taatgccACT	ATTTTATTGA	060
	TATGTAGTTT	TATTAGTAAA	TAAGTATATC	TGTTCTATAA	CTATAAACTT	ATTGATTGTG	120
	AATACATATT	TTCTTAAAGA	TTTAAGTAAA	ATGTAATTTT	TTATAAACCA	CAGTCTTATT	180
	TGAGGGAATG	TAGAGACAAG	TGGGAGGCAG	TGTATTTGAA	GATACATTTA	ACTTGGGAGA	240
	TTGAAAACAT	TTTCAATTTTT	CTTTTTTTCT	CCCTTCATTG	CTTAACACAT	TTTCCTATTT	300
EXON 12	TTATCCCCTC	TAGGACTGTT	ATGAACACTA	TCCAACAATT	AATGATGATT	TAAATTCAG	360
		gThrVal	MetAsnThrI	leGlnGlnLe	uMetMetIle	LeuAsnSerA	(392)
	CAAGTGATCA	ACCTTCAGAA	AATCTGATTT	CCTATTTTAA	CGTAAGCCAT	ATATGAAACA	420
	laSerAspGl	nProSerGlu	AsnLeuIleS	erTyrPheAs	n		(405)
	TTATTTATTG	TAATATCTTG	GCAAAGAAAC	TTGAAATTAA	AAGTTAAAGT	ACTGAGTTCT	480
	TTTTAAAATA	CTAATCTCCT	ATCTAACATG	TAGTTATCCA	TAATCTTTTC	TTGCTTTTTT	540
	AATCTTACAA	ATTATATATT	ATTAGTAGTA	TTGTTTTATT	TATACAGTGT	TATTTAAAAC	600
	ATTTTTATGT	TTACCTATTT	GCCTTgctca	ccattcttcc	ttcgaactta	tgccctcactt	660
	ctgagataat	tttttcttct	tcagatatat	ccttttgataa	ttac		704
	--3.1 kb--						
	aaaattttaga	taatagggtt	ttttagttgt	actgtagtat	tttttgctcg	attaacatcc	060
	AGTGAAATGA	TATTGTCTGC	TTATGTTTCAG	TAGTTGTGGT	TACCTAGTTA	TTATGGAAGT	120
	GTTTCCACAT	TTTTATGAAC	AATTTAAAAA	GTCATATATT	ATGGAGCAGA	AAATATTAAT	180
EXON 13	TCTGATTACA	CAGTATCCTC	GACATTGATT	TCTGTTTTTA	CCTCCTAAAG	AACTGCACAG	240
					AsnCysThrV		(409)
	TGAATCCAAA	AGAAAGTATA	CTGAAAAGAG	TGAAGGATAT	AGGATACATC	TTTAAAGAGA	300
	alAsnProLy	sGluSerIle	LeuLysArgV	allLysAspIl	eGlyTyrIle	PheLysGluL	(429)
EcoRI	AATTTGCTAA	AGCTGTGGGA	CAGGGTTGTG	TCGAAATTGG	ATCACAGGTA	ACTTGAATTC	360
	ysPheAlaLy	sAlaValGly	GlnGlyCysV	alGluIleGl	ySerGln		(444)
	ATTGTAATTC	GTGGTACTAT	AGAGTAATAA	TATTAAAAGC	AGCATCTTTC	CAGTTCGTAT	420
	AAATACTCTA	ACAGTATTTG	TCTAGTAGTA	TAAAATACTG	TCAGATACTA	TATCCCTGCT	480
	GCCTGTGTAT	GCTGCTATTT	ATGGGAACTT	TATGGAAAAC	TACCTCCCAc	cccattataa	540
	aaactatgta	ataaaggaac	acatagccat	tgtagaaatt	ttng		584
	--1.8 kb--						

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FIG. 6-5

	gaatgttaat	caccacttaa	tacttaagtt	gtgagtttta	gacaagctng	cttttgtgtt	060
	gtcttggegg	ccatatttgt	agaaggggtg	AGAAGTATGT	TTTAAGAAAA	GGCTTTTAA	120
	AAAATTTTAG	TAATTGTCAG	CTGGGTATAG	TGGTACATGC	CTATAATCCC	AGCCTCTTGG	180
	GAGGCCAAAG	CAGGAGGATC	TCTTGAGCCC	AGGAGTGTGA	AGGCCAGCCT	GGGCAAAACA	240
	GTGAGACTCC	ATCTCAAAAA	AAAAAAAAAA	TTTCATAATT	GTGATTTTCT	AAAATAGCAG	300
EXON 14	GCTCTTATTI	TTCTTTTTGT	TTGTTTGTAG	CGATACAAAC	TTGGAGTTTC	CTTGTATTAC	360
				ArgTyrLysL	euGlyValAr	gLeuTyrTyr	(454)
	CGAGTAATGG	AATCCATGCT	TAAATCAGTA	AGTTAAAAAC	AATAATAAAA	AAATTTTCANC	420
	ArgValMetG	luSerMetLe	uLysSer				(463)
	CGGGCGCGGT	GGCTCACGCC	TGCAATCCCA	GCACTTTGGG	AGGCCGAGGT	GGGCAGATCA	480
	GGAGGTCAAG	GCATCAAGAT	CATCCTGGCC	AAAATGGTGA	AACCCTGTCT	CTACTAAAAAG	540
	TACAAAAATT	AGCTGGGCGT	GGTGGTGTAG	ACCTGTAGTC	CCAGCTACTT	GGCAGGCTGA	600
	GGCAGGAGAA	TCCCTTGAAC	CACGGAGGTG	GAGGTTGCAG	TGAGCCAAGA	TTGTGCCATT	660
	TCACCCACAG	CTGGCAACAG	AGCAAGACAC	CATCTAAAAA	AAAAAAAAAA	AAAAAAAAAA	720
	ATTCAATGCT	GACACAAATA	AGGTTTCAAT	TAAACAACCT	CTTTTTTTTT	TTTAAATTA	780
EXON 15	TCTGTTTCAG	GAAGAAGAAC	GATTATCCAT	TCAAAATTTT	AGGTAAATTT	TTTACTTTTA	840
		GluGluGluA	rgLeuSerIl	eGlnAsnPhe	Se		(474)
	GTAAAAAATT	TTTTTCTTTT	TATAGAAGTA	AGTATTTTAT	AATCTTTTTT	TTTTTCCTTT	900
EXON 16	AGCAAACTTC	TGAATGACAA	CATTTTTCAT	ATGTCTTTAT	TGGCGTGCGC	TCTTGAGGTT	960
	rLysLeuL	euAsnAspAs	nIlePheHis	MetSerLeuL	euAlaCysAl	aLeuGluVal	(493)
	GTAATGGCCA	CATATAGCAG	TAAGTTAAAT	TTTCATAAAT	AAACACTTTT	GTTCAATTTA	1020
	ValMetAlaT	hrTyrSerA					(500)
	AAGTTAAAT	GTGGTGTGTT	TCTTTGGTCT	GGGGAGAGGG	ATAGTGTGAG	GTAAAGGAGA	1080
	AGGAATGCTT	ATTTTAGATC	ACTATATACT	GAAGAATGTA	ATTGGTCATT	ATAAGCCATT	1140
	TAAGAGGCTT	ATTTGAGTTA	TTTGAggcca	tcttggggat	aatattttcac	taggcttctc	1200
	ttctgagtat	actggtatac	tgaatccaaa	aaaggtactt	tttcgaaatc	cctccgaaga	1260
	ccttttgagat	tgtagagtgc					1280
				--1.0 kb--			
	ggtattttaaa	tcttttgaaaa	tttgagatca	gctataagtc	ctttctctag	gaaaaacaca	060
	gaTTTGCATA	CACTCAAAAT	TGGAAGGCTA	TTTCCTATGA	GTCCGTAGAC	TCCAAAATAA	120
	AAAATTCTGC	TCTAAATAAA	AATGGTTTAA	CCTTTCTACT	GTTTTCTTTG	TCTGATAATA	180
	ACTTCCAAAA	AAATACCTAG	CTCAAGGGTT	AATATTTTCAT	AAATAGTTAC	TTTTTTTTTT	240
EXON 17	CATTTTTAGG	AAGTACATCT	CAGAATCTTG	ATTCTGGAAC	AGATTTGTCT	TTCCCATGGA	300
		r gSerThrSer	GlnAsnLeuA	spSerGlyTh	rAspLeuSer	PheProTrpI	(517)
	TTCTGAATGT	GCTTAATTIA	AAAGCCTTTG	ATTTTTACAA	AGTGATCGAA	AGTTTTATCA	360
	leLeuAsnVa	lLeuAsnLeu	LysAlaPheA	spPheTyrLy	sValIleGlu	SerPheIleL	(537)
	AAGCAGAAGG	CAACTTGACA	AGAGAAATGA	TAAAACATTT	AGAACGATGT	GAACATCGAA	420
	ysAlaGluGl	yAsnLeuThr	ArgGluMetI	leLysHisLe	uGluArgCys	GluHisArgI	(557)
	TCATGGAATC	CCTTGCATGG	CTCTCAGTAA	GTAGCTAAAT	AATTGAAGAA	ATTCATTCAT	480
	leMetGlySe	rLeuAlaTrp	LeuSer				(565)
	GTGCATATGG	CTAACAAATT	ATTGTTAGTG	AGAGGTGTTT	CTTAACAAAT	CTACCTCAAG	540
	AACAAATAGG	GAATTIAATG	AATAATGTIA	TTTCAGTCTA	TAGCCCAAGG	ATCAagtgga	600
	atattagaat	ggagcttttaa	tcgagcacc	ttaaaccatct	aatacagcnc	agtgatttat	660
	ttaagaatag	cttttctttaa	aacatgccac	ttt			693
				--70.0 kb--			

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FIG. 6-6

EXON 18	atttttcta	ataagcg	aaggttata	atttttctac	ttttttgtgt	gtgggaagta	060
	caaaaattgt	CAATTGGGAA	TTTCGAAGTA	GAGAAAAATA	TTTCATTCTG	ACTTTTAAAT	120
	TGCCACTGTC	AATTGTGCCT	AAAATTCATA	GTACTTACCA	TGTCAAACAA	TATGATTTTG	180
	ATATGTACCT	GGGAAAATTA	TGCTTACTAA	TGTGGTTTTA	ATTTTCATCAT	GTTTCATATA	240
	GGATTCACCT	TTATTTGATC	TTATTAAACA	ATCAAAGGAC	CGAGAAGGAC	CAACTGATCA	300
	AspSerPro	LeuPheAspL	euIleLysG1	nSerLysAsp	ArgGluGlyP	roThrAspHi	(585)
	CCTTGAATCT	GCTTGTCCCT	TTAATCTTCC	TCTCCAGAAT	AATCACACTG	CAGCAGATAT	360
	sLeuGluSer	AlaCysProL	euAsnLeuPr	oLeuGlnAsn	AsnHisThrA	laAlaAspMe	(605)
	GTAAGCAAAA	TATATGTTAT	GTTGACCATC	AAACTGCAAA	TAGATTTTAA	GCATAAGTGC	420
	AATGTAACAT	TCTATAAAGA	AAGTGTAGGG	AATAGAATTT	TGAATAAGAA	TAGTTTCTGT	480
	TTTAAAGAAA	TTAGTAATAA	AAGGTACATg	acccaaataa	agtcataataa	aagagtacag	540
	agtgtactg	aatcacctag	gatttgcata	atgagagcag	ttttcatgg		589
	--3.0 kb--						
EXON 19	tgtttttaag	ctggaatcac	cttatgggtct	caataccact	ataattatta	aaattgTACA	060
	TTATACATAT	ATAGCTATTT	TTTTCTAATA	AGGCAGTAAT	CCCCAGGAAA	AGCCATTTAT	120
	TAAAATAGAA	TTAGATATGA	TGATGACAAG	CAGTTTTCTG	ATTAATATAT	CTTCCCAGC	180
	TTGCATTAA	ATAGTCTGCT	ATAATACCAA	TTAAATAGAC	AAGATGTATC	TGGGTGTACA	240
	ACCTTGAAGT	GTATGTATAA	TCTGTGATTG	TTAGCCAACT	TGAAATGAAG	ACTTTTCTTT	300
	TAAATATATC	TAGGTATCTT	TCTCCTGTAA	GATCTCCAAA	GAAAAAAGGT	TCAACTACGC	360
		tTyrLeu	SerProValA	rgSerProLy	sLysLysGly	SerThrThrA	(621)
	GTGTAAATTC	TACTGCAAAT	GCAGAGACAC	AAGCAACCTC	AGCCTTCCAG	ACCCAGAAGC	420
	rgValAsnSe	rThrAlaAsn	AlaGluThrG	lnAlaThrSe	rAlaPheGln	ThrGlnLysP	(641)
	CATTGAAATC	TACCTCTCTT	TCAGTGTCTT	ATAAAAAAGG	TTAGTAGATG	ATTATTTTCA	480
	roLeuLysSe	rThrSerLeu	SerLeuPheT	yrLysLysV			(654)
	AGAGCATGGA	CTCTGAAACT	AGGCTGACTG	GGTTCAAATC	ATGTTTCTTC	TACTTTCTAG	540
	GTACATTACT	GGGCAAGTCA	CTTAATATCT	CTGTGTCTCA	GTTTCCTcat	ctataaaatg	600
	gaaatgataa	tgttgcgaga	tctttcttga	ctattcagag	tcgttttctg		650
	--2.8 kb--						
EXON 20	aaggaaaatc	catgccccnt	cgggacatgc	ctgncctctg	cattttcttea	tctgtatccc	060
	ttgtaatatg	cctcataata	aaccagtaaa	catGTTTCTC	TGGGGGAAAG	AAAAGAGTGG	120
	TAGAAAAGAG	GTTTCTGTTA	AAATGCTACT	TAACAGCATT	ATAATTAGTG	TAATTTTCATG	180
	ATTTGAAAAA	AATCTACTTG	TAATTCAAAA	TGAACAGTAA	AAATGACTAA	TTTTTCTTAT	240
	TCCCACAGTG	TATCGGCTAG	CCTATCTCCG	GCTAAATACA	CTTTGTGAAC	GCCTTCTGTC	300
		al TyrArgLeuA	laTyrLeuAr	gLeuAsnThr	LeuCysGluA	rgLeuLeuSe	(671)
	TGAGCACCCA	GAATTAGAAC	ATATCATCTG	GACCCTTTTC	CAGCACACCC	TGCAGAATGA	360
	rGluHisPro	GluLeuGluH	isIleIleTr	pThrLeuPhe	GlnHisThrL	euGlnAsnG1	(691)
	GTATGAAGTC	ATGAGAGACA	GGCATTGGA	CCAAGTAAGA	AAATCAAGCA	CTTCACCTTC	420
	uTyrGluLeu	MetArgAspA	rgHisLeuAs	pGln			(702)
	TCTCCTCCCT	ACTTACTTGT	TAAGTGATTT	CTTTCTTTCT	TTCTTTCTTT	CTTTCTTTCT	480
	TTCTTTCTTT	CTTTCTTTCT	TTCTTTCTTT	TCTTTCTTTT	CTTTCTTTCT	TTCCTTTTTT	540
	TTTTTTTGAG	ATAGAGTCTC	ACTCTGTTAC	CCAGGCTGGA	GTGCAGTGGC	GCAATCTCGG	600
	CTCACTGCAA	CCTCCGCCTC	CCAGGTCAAG	TGATTCTCCT	GCCTCAGCCT	ccnaggagct	660
	aggatacagg	cgtgtaccac	cacaccttgt	taatttttgt	tatttagtag	agacagg	717
	--4.0 kb--						

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FIG. 6-7

	caagagccaa	agttagggtg	atttacaac	caggtgatca	gtcctggata	attgagcctt	060
	ggtgatttgC	ATTTTGTCT	TTAAACACAC	TTTGGGTAA	ACACTTCATG	TAGACTTTCA	120
<u>SacI</u>	AACTGAGCTC	AGTATGGAAA	GAAATAACTC	TGTAGATTAA	ACCTTTCTTT	TTTGAGGCTA	180
	AAAGAAAGAA	AATGGTATTT	TTTAAGAACA	AAACCATGTA	ATAAAATTCT	GACTACTTTT	240
EXON 21	ACATCAATTT	ATTACTAGA	TTATGATGTG	TTCCATGTAT	GGCATATGCA	AAGTGAAGAA	300
		I	leMetMetCy	sSerMetTyr	GlyIleCysL	ysValLysAs	(716)
	TATAGACCTT	AAATTCAAAA	TCATTGTAAC	AGCATACAAG	GATCTTCCTC	ATGCTGTTCA	360
	nIleAspLeu	LysPheLysI	leIleValTh	rAlaTyrLys	AspLeuProH	isAlaValGI	(736)
	GGAGGTAGGT	AATTTTCCAT	AGTAAGTTTT	TTTGATAAAT	CCATATCCAT	AACATAACAT	420
	nGlu						(737)
	AGGTAATTCA	TTTGATCTCA	TTTATCATT	ATGAGATCAT	ATATTCTGTC	TGACCTTATT	480
	ATGTAAATTC	ACAAATAAAA	ACTTTTATAT	TATTTATTG	TAACTTAAAT	AGAATTGGAA	540
	AGATAAGGGT	AATTATGAAA	TTACCCATAT	CATAGTTTTT	TATAAAGTTA	ATAAATAATA	600
	TTTTATCCCT	GTAATAAGCA	GGTATTTGTa	ataaacttga	catgagtcac	agaacattag	660
	atatccttgag						670
				--0.2 kb--			
<u>XbaI</u>	tccatctgct	gctgcctggc	tatttctctc	aatcgattct	gtgacatttc	acttctagaa	060
	gagcaGCTAT	AATCCAAGCC	TAAGAAGTAA	TTTTATTAT	TTATTATTTT	TTCCTTTATA	120
	ATATGTGCTT	CTTACCAGTC	AAAAAGTATT	ATAAACTATT	AGAAAAGAAA	ATCTAAAGGT	180
	AGAAATTTTA	AAATTCATTT	AACAAGTAAA	TTTTACTTTT	TTTTTTTTTT	TTTTTTTTTT	240
EXON 22	ACTGTTCTTC	CTCAGACATT	CAAACGTGTT	TTGATCAAAG	AAGAGGAGTA	TGATTCTATT	300
		ThrPh	eLysArgVal	LeuIleLysG	luGluGluTy	rAspSerIle	(752)
	ATAGTATTCT	ATAACTCGGT	CTTCATGCAG	AGACTGAAAA	CAAATATTTT	GCAGTATGCT	360
	IleValPheT	yrAsnSerVa	lPheMetGln	ArgLeuLysT	hrAsnIleLe	uGlnTyrAla	(772)
	TCCACCAGGG	TAGGTCAAAA	GTATCCTTTG	ATTGGAAAAA	TCTAATGTAA	TGGGTCCACC	420
	SerThrArg						(775)
EXON 23	AAAACATTAA	ATAAATAATC	TACTTTTTTG	TTTTTGCTCT	AGCCCCCTAC	CTTGTCACCA	480
					ProProTh	rLeuSerPro	(781)
	ATACCTCACA	TTCCTCGAAG	CCCTTACAAG	TTTCCTAGTT	CACCCCTACG	GATTCCTGGA	540
	IleProHisI	leProArgSe	rProTyrLys	PheProSerS	erProLeuAr	gIleProGly	(801)
	GGGAACATCT	ATATTTCCAC	CCTGAAGAGT	CCATATAAAA	TTTCAGAAGG	TCTGCCAACA	600
	GlyAsnIleT	yrIleSerPr	oLeuLysSer	ProTyrLysI	leSerGluGl	yLeuProThr	(821)
	CCAACAAAAA	TGACTCCAAG	ATCAAGGTGT	GTGTTTTCTC	TTTAGGGAAG	TAGTAAAGAA	660
	ProThrLysM	etThrProAr	gSerAr				(830)
	TGAGAGGGGG	ATTATTTTGA	TCCAAGAATA	AAAAATATAA	AGCATTCTTC	ATTTCAAATA	720
	AGCTAGACTC	TTGAAACTCT	ATTTGCTTAT	TTAAGTAACA	TAATAAGAAT	ATGGGGGGCG	780
	GGTGAAGAAA	ATCTATTTAC	GACTTAAGCA	ACGCAAGATG	GCCGAATAGG	AACAGCTCCg	840
	gtctacagct	cccagcgtga	gcacgcagaa	gacgggtgat	ttctgcattt	ccatctgagg	900
	taccgggttc	atctcactag	ggagtgccag	acagt			935
				--7.4 kb--			
	ttgataactt	accattgat	ttatgaagaa	ctaagtaggg	gtaaccttga	aacttgctt	060
	tgccctccct	aatatggggc	aatggcagna	tatgttcttg	cagacctata	acttttgctT	120
	TAAACTAAG	AGACTAGGTG	AGTATATGAT	TAGACGGGCA	CTGTTAGAAT	AATCCCCAAA	180
	TGAATATAGT	TTGTCAGTGG	TTCTAGGGTA	GAGGTAACCT	TTAATTTGGT	ATTCCTAATA	240
	GTTTCAAGATG	ATGTATTTAT	GCTCATCTCT	GCAAAATTGT	ATATGGTTTT	TTATTACTAA	300
EXON 24	TTGGTATTTT	ATCTTAACTT	GACAGAATCT	TAGTATCAAT	TGGTGAATCA	TTGGGGGTGA	360
		gIleL	euValSerII	eGlyGluSer	PheGly		(840)
<u>HindIII</u>	GTATTTTCTT	TCTATGAAAT	ATAATAGTAT	GCATTGTAAG	TATAAAAGAA	ATTAAAGCTT	420
	TCTATAATTT	GAATTTCCAA	ATGCAGTTAT	TCAAACACCT	CATCCAGGCA	TATTGCATAG	480
	AATTTTATGA	GATATATATA	TCTCAGATTT	ACTTTCAAAT	CAAGTTTAAT	CTCAAATCAT	540
	ACTCCTAATT	GGTGAACCTC	AAAACCTTTT	TAAATATCCA	CTTGAGATTA	TATAATACAT	600
	ATATACATTT	GTGTATATAC	ATACATATAT	ACGTGAGCTG	TTTTTGCTCA	CAACATTTCT	660
	ATCACCAAAT	GTGTGAGATT	TTTTTCTCAC	CCAAATCTAT	TCTTcaactc	tctgggtgctt	720
	ctacaattca	attcaattct	gacactaatt	accagag			758
				--2.8 kb--			

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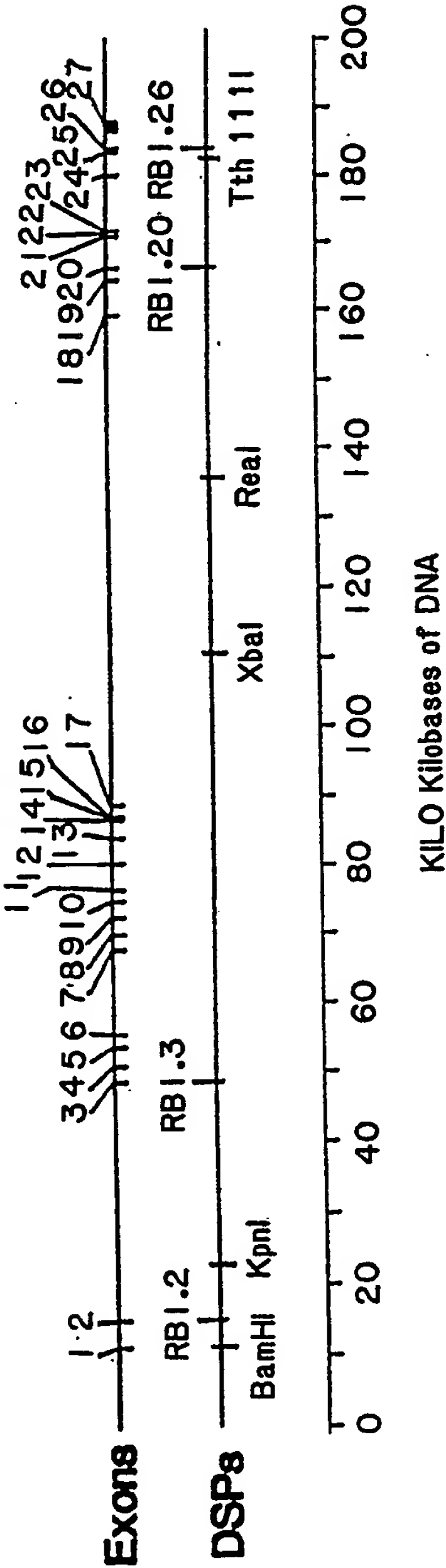
FIG. 6-8

ggggatggaa ttaggtagtt attctgattt ttAGATTTTT CATATCTTTT ATTTGGTCCA 060
 ATGAAGCAGA AAATTTAAAT GAAGTTATTA CCTTGCCTG ATTTTGGACA CACCTCAAAC 120
 EcoRI TATAACTTGA GGTGCTAAC TATGAAACAC TGGCATTAA TGATTAAAG TAAAGAATTC 180
 EXON 25 TGTAATTGT AGACTTCTGA GAAGTTCCAG AAAATAAATC AGATGGTATG TAACAGCGAC 240
 ThrSerGln uLysPheGln LysIleAsnG InMetValCy sAsnSerAsp (856)
 CGTGTGCTCA AAAGAAGTGC TGAAGGAAGC AACCTCCTA AACCACTGAA AAAACTACGC 300
 ArgValLeuL ysArgSerAl aGluGlySer AsnProProL ysProLeuLy sLysLeuArg (876)
 TTTGATATTG AAGGATCAGA TGAAGCAGAT GGAAGGTAGG AACCACTTTT GAATGTTTTT 360
 PheAspIleG luGlySerAs pGluAlaAsp GlySe (888)
 CAGTAGCTGA GATGGTCATC TGGGGAATCC AGAGTCTCAG CACTGCTCCT GGCTTATACC 420
 AATTTCTTTC ATGCCAAGTT TATTTGGAAG TTGTGAGAAT GGCTCAAAAT AATAGATATG 480
 AGTGTAGTGC AAAGTTAAAA ACATCTTACA AATTGCATAC CAACATTCAG TGAAGATATC 540
 TAATAAACCCT TGATCTTTTT TACAAAGCTA TTGATAAAAT TTTGTTATTC TTAACATTAA 600
 ATTTAAAAAT GTTTACTCTT GAAAAATATT AACCACTGTA TTTTGTGAGA ACCACTGAAA 660
 AAATACATAG CATCATAAAT TTGTGACATT TATGTTTTAG ATGGTTAGTT TTTAAATTTT 720
 AAAATTAAAA GCTACTCACT AAAATAATAG CATAAAGTAA GTCATCGAAA GCATCATAGT 780
 TACTGGAAAT TTGAGTTTTT CATTATATAA TACACATGAA ATGTTTTGCA TTTTTTTAAT 840
 EXON 26 CTGCAGTAAA CATCTCCAG GAGAGTCCAA ATTTGAGCAG AAAGTGGCAG AAATGAGTAA 900
 rLys HisLeuProG lyGluSerLy sPheGlnGln LysLeuAlaG luMetT (905)
 GTACTTTTTT CACCTTGTGT AAACGAAATA AACAAATTGT TACACTGCAA GAAGTCTTTT 960
 CGTTATATAA AAGAATGTAT AATTTCTTCA GTTGGCAGGT TTGTTTATGC ATTTAAATA 1020
 TAATTCAATC AAGGTATTTT ATCTACAAAC ATTTGTGGAT TAAATGTATG ATGTAAATG 1080
 AAGGTCATTT TTACCTTTT TATGATCTTT CATGCAGGAA GACTAAGAAG TGAAACATTG 1140
 CTTGACCACA TTCAAcacaa atggctacag ttagaaaata ctttagcaga actacaaaga 1200
 ggaactattt gggagtgtta gatataggga aaagttttat aaacctagca tatgtaaaac 1260
 atcatcacc ttatttsagg aataaccttt gattctaccg atttttaaac 1310
 --1.7 kb--
 tctagctatt tgaatatgca gtaaattaac tgtaactcct acggtactgt caaatactag 060
 aatgaagacc acctcttttt gcaAGGTCCT GAGCGCCATC AGTTTGACAT GAGCATAATA 120
 TATATGGCAG CCACTTGCCA ACTTACCCAG TACCATCAAT GCTGTTAACA GTTCTTCATC 180
 EXON 27 CTTTTTCCAG CTTCTACTCG AACACGAATG CAAAAGCAGA AAATGAATGA TAGCATGGAT 240
 hrSerThrAr gThrArgMet GlnLysGlnL ysMetAsnAs pSerMetAsp (921)
 ACCTCAAACA AGGAAGAGAA ATGAGGATCT CAGGACCTTG GTGGACACTG TGTACACCTC 300
 ThrSerAsnL ysGluGluLy sEND 3 untranslated region begins here (928)
 TGGATTCATT GTCTCTCACA GATGTGACTG TATAACTTTC CCAGGTTCTG TTTATGGCCA 360
 CATTTAATAT CTTGAGCTCT TTTTGTGGAT ATAAAATGTG CAGATGCAAT TGTTTGGGTG 420
 ATTCCTAAGC CACTTGAAAT GTTAGTCATT GTTATTTATA CAAGATTGAA AATCTTGTGT 480
 AAATCCTGCC ATTTAAAAAG TTGTAGCAGA TTGTTTCCTC TTCCAAAGTA AAATTGCTGT 540
 GCTTTATGGA TAGTAAGAAT GGCCCTAGAG TGGGAGTCCT GATAACCCAG GCCTGTCTGA 600
 CTACTTTGCC TTCTTTTGTA GCATATAGGT GATGTTTGCT CTTGTTTTTA TTAATTTATA 660
 TGTATATTTT TTTAATTTAA CATGAACACC CTTAGAAAAT GTGTCCTATC TATCTTCCAA 720
 ATGCAATTTG ATTGACTGCC CATTACCAA AATTATCCTG AACTCTTCTG CAAAAATGGA 780
 TATTATTAGA AATTAGAAAA AAATTACTAA TTTTACACAT TAGATTTTAT TTTACTATTG 840
 GAATCTGATA TACTGTGTGC TTGTTTTATA AAATTTTGCT TTTAATTAAA TAAAAGCTGG 900
 AAGCAAAGTA TAACCATATG ATACTATCAT ACTACTGAAA CAGATTTTCAT ACCTCAGAAT 960
 GTAAAAGAAC TTAAGTATTA TTTTCTTCAT CCAACTTATG TTTTAAATG AGGATTATTG 1020
 ATAGTACTCT TGGTTTTTAT ACCATTGAGA TCACTGAATT TATAAAGTAC CCATCTAGTA 1080
 CTTGAAAAAG TAAAGTGTTT TGCCAGATCT TAGGTATAGA GGACCCTAAC ACAGTATATC 1140
 CCAAGTGCAC TTTCTAATGT TTCTGGGTCC TGAAGAATTA AGATACAAAT TAATTTTACT 1200
 CCATAAACAG ACTGTTAATT ATAGGAGCCT TAATTTTTTT TTCATAGAGA TTTGTCTAAT 1260
 TGCATCTCAA AATTATCTG CCCTCCTTAA TTTGGGAAGG TTTGTGTTTT CTCTGGAATG 1320
 GTACATGTCT TCCATGTATC TTTTGAACCT GCAATTGTCT ATTTATCTTT TATTTTTTTA 1380
 AGTCAGTATG GTCTAACACT GGCATGTTCA AAGCCACATT ATTTCTAGTC CAAAATTACA 1440
 HindIII AGTAATCAAG GGTCAATTAT GGTAGGCAT TAATGTTTCT ATCTGATTTT GTGCAAAAGC 1500

FIG. 6-9

EXON 27 3' untranslated region (continued)
(CONTD) TTCAAATTAA AACAGCTGCA TTAGAAAAG AGGGCTTCT CCGCTCCCCT ACACCTAAAG 1560
GTGTATTAA ACTATCTTGT GTGATTAACT TATTAGAGA TGCTGTAACT TAAAATAGGG 1620
GATATTTAAG GTAGCTTCAG CTAGCTTTTA GGAAATCAC TTGTCTAAC TCAGAAATTAT 1680
TTTTAAAAG AAATCTGGTC TTGTTAGAAA ACAAAATTTT ATTTTGTGCT CATTTAAGTT 1740
TCAAACTTAC TATTTTGACA GTTATTTTGA TAACAATGAC ACTAGAAAAC TTGACTCCAT 1800
TTCATCATTTG TTTCTGCATG AATATCATAC AATCAGTTA GTTTTTAGGT CAAGGGCTTA 1860
CTATTCTGG GTCCTTTGCT ACTAAGTTCA CATTAGAAAT AGTGCCAGAA TTTTAGGAAC 1920
TTCAGAGATC GTGTATTGAG ATTCTTAAA TAATGCTTCA GATATTATTG CTTTATTGCT 1980
TTTTTGTATT GGTAAAACT GTACATTTAA AATTGCTATG TTACTATTTT CTACAAATTAA 2040
TAGTTTGTCT ATTTTAAAT AAATTAGTTG TTAAGAGTC TTAATGGTCTG ATGTTGTGTT 2100
polyadenylation signal sequence polyadenylation site
CTTTGTATTA AGTACACTAA TGTCTCTTT TCTGTCTAGG AGAAGATAGA TAGAAGATAA 2160
CTCTCCTAGT ATCTCATCCA TTCCTAGCCT TTAAGGGCT CTATATGCTA GAGATTTCCTA 2220
AATTATTTG TTCAGCCCTG ATCTTTTCAC AGAGTCAAG Gctttttag ccaacagaac 2280
tcttgattcc tactccntc taccaatgt ctccaaatat aaactaaat caaataaata 2340
aaaatctttt tt 2352

FIG. 7
HUMAN RETINOBLASTOMA GENE



RB-6

FIG. 8

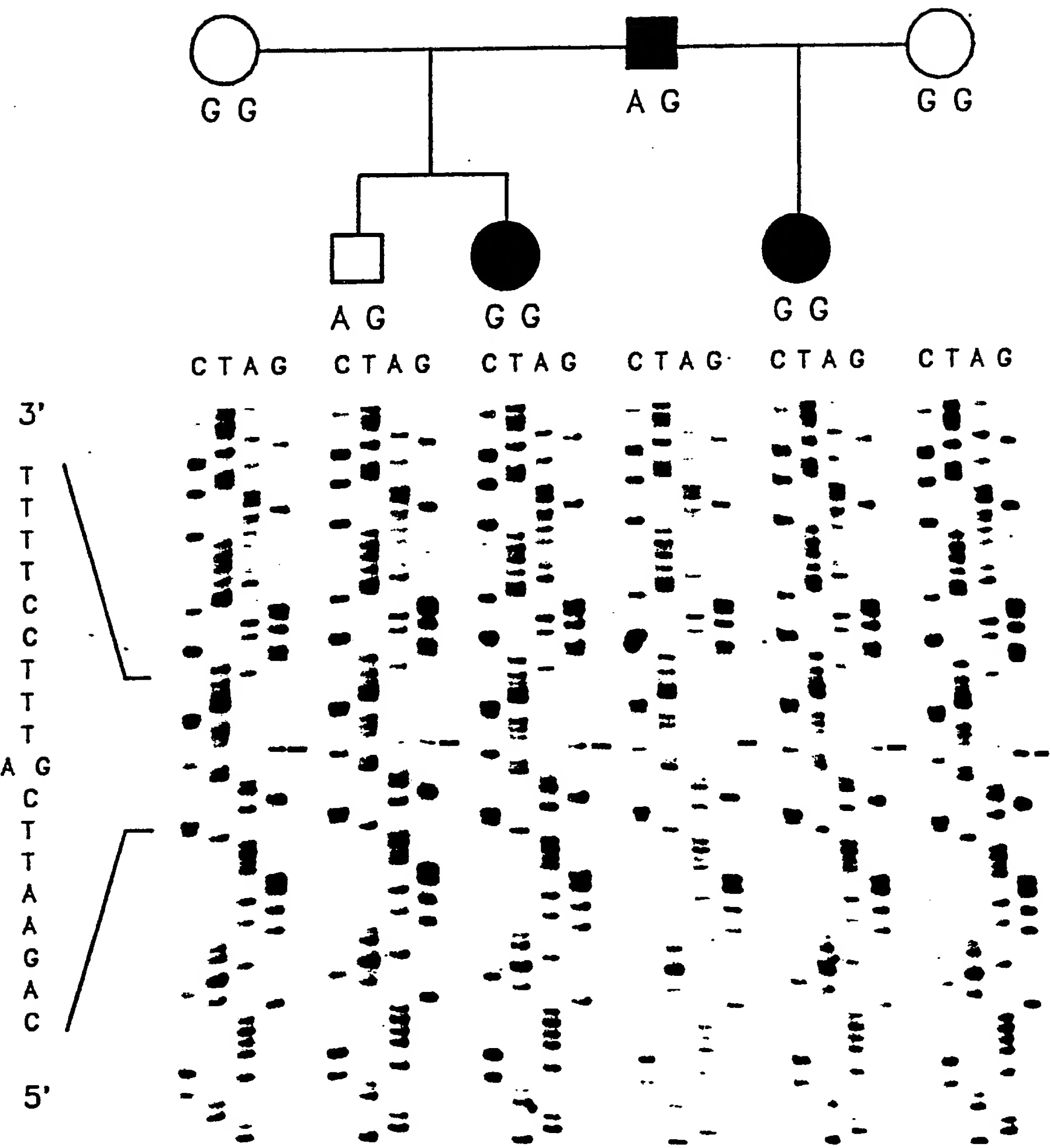
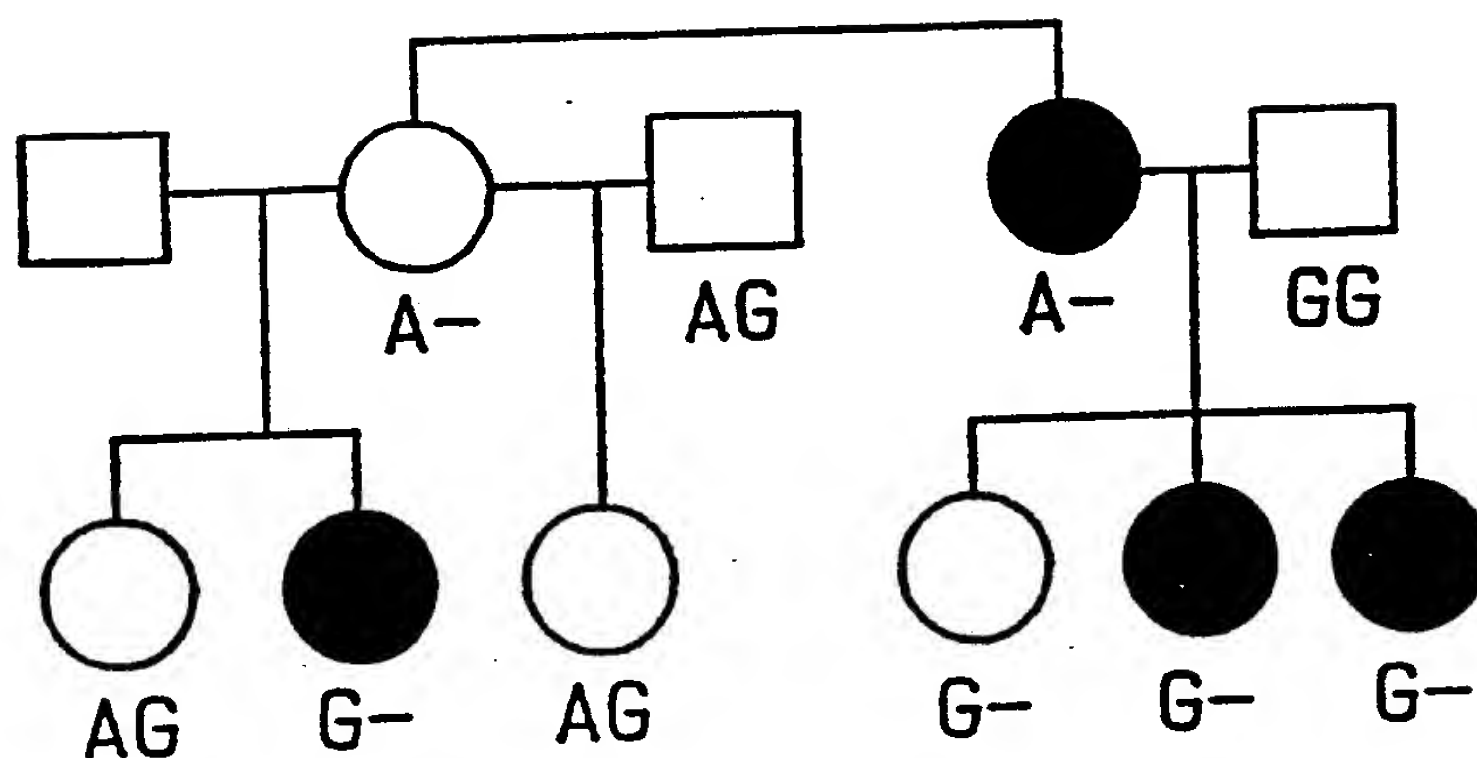
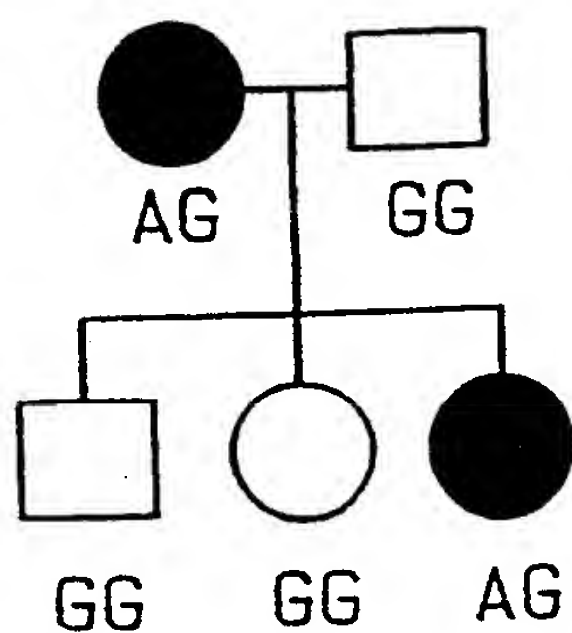


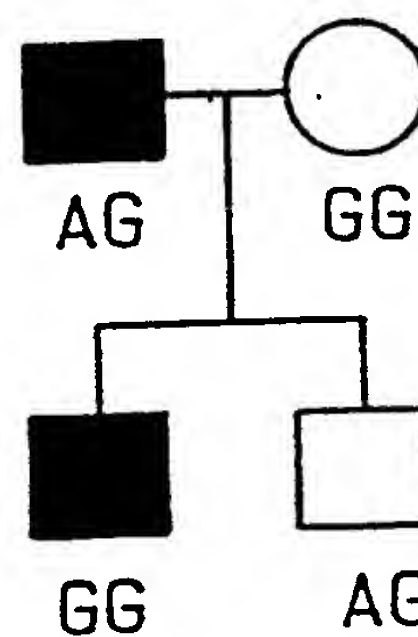
FIG. 9 RB-32



RB-36



RB-50



INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US89/00293**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC <div style="display: flex; justify-content: space-between; margin-top: 5px;"> IPC(4): C12Q 1/68 G01N 30/00 U.S.: 435/6 536/27 </div>																	
II. FIELDS SEARCHED <div style="text-align: center; margin-top: 5px;">Minimum Documentation Searched ⁷</div> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <th style="width: 20%;">Classification System</th> <th>Classification Symbols</th> </tr> <tr> <td style="text-align: center; vertical-align: middle;">U.S.</td> <td style="text-align: center; vertical-align: middle;">435/6 536/27</td> </tr> </table> <div style="text-align: center; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div> <div style="margin-top: 10px;">APS; CAS; BIOSIS DATABASES</div>			Classification System	Classification Symbols	U.S.	435/6 536/27											
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U.S.	435/6 536/27																
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <th style="width: 10%;">Category [*]</th> <th style="width: 70%;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td>SCIENCE, Volume 235 issued 13 March 1987 (Washington, D.C. USA) W. LEE "Human Retinoblastoma Susceptibility Gene: Cloning, Identification and Sequence" entire article.</td> <td style="text-align: center; vertical-align: top;">1,4,8-16</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">P,X</td> <td>EP, 0,259031 (Massachusetts Eye and Ear Infirmary) 09 March 1988</td> <td style="text-align: center; vertical-align: top;">1,4,8-16</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td>Chemical Abstracts, Volume 107, No. 9 issued August 1987 (Columbus, Ohio, USA) S.A. LATT "DNA based detection of chromosome deletion & amplification" abstract No. 71579; Cold Spring Harbor Symp. Quant. Biol. 51(1) 299-307</td> <td style="text-align: center; vertical-align: top;">1,4,8-16</td> </tr> <tr> <td style="text-align: center; vertical-align: top;"><u>X</u> Y</td> <td>Chemical Abstracts, Volume 106 No. 7, issued February 1987 (Columbus, Ohio, USA) H. Scheffer, "A straightforward approach to isolate DNA sequences with potential linkage to the retinoblastoma locus" abstract No. 449462, Hum. Genet. 74(3) 249-255</td> <td style="text-align: center; vertical-align: top;"><u>14</u> 8-13,15,16</td> </tr> </table>			Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	SCIENCE, Volume 235 issued 13 March 1987 (Washington, D.C. USA) W. LEE "Human Retinoblastoma Susceptibility Gene: Cloning, Identification and Sequence" entire article.	1,4,8-16	P,X	EP, 0,259031 (Massachusetts Eye and Ear Infirmary) 09 March 1988	1,4,8-16	Y	Chemical Abstracts, Volume 107, No. 9 issued August 1987 (Columbus, Ohio, USA) S.A. LATT "DNA based detection of chromosome deletion & amplification" abstract No. 71579; Cold Spring Harbor Symp. Quant. Biol. 51(1) 299-307	1,4,8-16	<u>X</u> Y	Chemical Abstracts, Volume 106 No. 7, issued February 1987 (Columbus, Ohio, USA) H. Scheffer, "A straightforward approach to isolate DNA sequences with potential linkage to the retinoblastoma locus" abstract No. 449462, Hum. Genet. 74(3) 249-255	<u>14</u> 8-13,15,16
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <td style="width: 50%; vertical-align: top;"> Date of the Actual Completion of the International Search <div style="text-align: center; font-size: 1.2em;">29 April 1989</div> </td> <td style="width: 50%; vertical-align: top;"> Date of Mailing of this International Search Report <div style="text-align: center; font-size: 1.5em;">08 JUN 1989</div> </td> </tr> <tr> <td style="vertical-align: top;"> International Searching Authority <div style="text-align: center;">ISA/US</div> </td> <td style="vertical-align: top;"> Signature of Authorized Officer <div style="text-align: center;">SCOTT A. CHAMBERS</div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center; font-size: 1.2em;">29 April 1989</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-size: 1.5em;">08 JUN 1989</div>	International Searching Authority <div style="text-align: center;">ISA/US</div>	Signature of Authorized Officer <div style="text-align: center;">SCOTT A. CHAMBERS</div>											
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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X

Chemical Abstracts, Volume 105, No.17,
issued October 1986 (Columbus, Ohio, USA)
E.Y. LEE, "Molecular cloning of the human
esterase D gene a genetic marker of
retinoblastoma" abstract No. 147520g,
Proc. Natl. Acad. Sci. U.S.A. 83(17)
6337-41.

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V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter^{1,2} not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out^{1,3}, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1, 4 and 8-16

II. Claims 2 and 3

III. Claims 5, 17 and 18

IV. Claims 6, 7 and 19-21

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1, 4 and 8-16

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Chemical Abstracts, Volume 102, No. 7 issued February 1985 (Columbus, Ohio, U.S.A.) M. LALANDE "Isolation of human chromosome 13-specific DNA sequences cloned from flow sorted chromosome and potentially linked to the retinoblastoma locus" abstract No. 57143C, Cancer Genet. Cytogenet 13(4) 283-95.	1,4,8-16